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PROCEEDINGS
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COMPARISON OF THE PHOSPHORUS CONTENT, OPTICAL ROTATION, SEPARATION OF HEMES AND GLOBIN, AND TERMINAL AMINO ACID RESIDUES OF NORMAL ADULT HUMAN HEMOGLOBIN AND SICKLE CELL ANEMIA HEMOGLOBIN

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Communicated by Linus Pauling, December 31, 1952

Introduction.—It has been shown that sickle cell anemia, a condition probably created[1, 2] by homozygosity in a single gene, is associated with the presence in the red blood cells of a hemoglobin different from normal adult hemoglobin.[1] Striking differences exist between the solubilities of the reduced forms of the two hemoglobins, the tendency of the abnormal hemoglobin to aggregate being the cause of the severe troubles in this molecular disease. Electrophoretic measurements revealed another characteristic difference; sickle cell anemia hemoglobin carries a greater positive charge or smaller negative charge in the pH range 5.5 to 8.0. In cases of sickle cell trait, where the sickling of the blood cells is brought about much more difficultly and no clinical troubles arise, electrophoretic analysis demonstrated the presence of normal and sickle cell anemia hemoglobin in roughly equal amounts. Apparently in these cases one dose each of the sickle cell gene and its normal allele are present in the somatic cell. A careful quantitative amino acid analysis of both hemoglobins pointed to an almost identical overall composition.[3] The isolation of the same porphyrin dimethyl ester from both hemoglobins indicates that the protein parts are largely responsible for the difference. There were indications of a slight difference in the content of leucine and serine and perhaps also of valine and threonine. These might account for the difference in electrophoretic behavior in an indirect manner. It was therefore decided to make a more detailed comparison of normal adult and sickle cell anemia hemoglobin, in order to disclose the chemical background of the hereditary abnormality. A full account of the electrophoretic behavior of the globins from normal adult hemoglobin, sickle cell

anemia hemoglobin, and sickle cell trait hemoglobin will be given in the following paper.[4] In this publication we report on some complementary studies concerning the phosphorus content, the optical rotary power, the splitting into heme and globin, and the terminal amino acid residues in both hemoglobins and globins.

Phosphorus Content.—We first checked whether phosphate groups might be responsible for the difference in charge of the molecules. It is reported in the older literature that hemoglobins of fowl contain a characteristic amount of phosphorus, whereas the hemoglobins of some mammals do not.[5, 6] We confirmed the latter fact for normal human hemoglobin. Thirty milligram samples of hemoglobin prepared according to Drabkin[7] were digested with 0.5 ml. conc. nitric acid and 0.5 ml. conc. sulfuric acid in 50-ml. Kjeldahl flasks. After completion of the destruction the sulfuric acid was carefully neutralized with 10 *N* sodium hydroxide. Solutions were decanted from solid sodium sulfate, diluted with water, and acidified with 4 *N* sulfuric acid, so that 6 ml. samples 0.5 *N* in sulfuric acid were obtained. These were colored with 0.5 ml. molybdate and 0.4 ml. aminonaphtholsulfonic acid solution as described by King.[8] Blanks, standards, and determinations with known amounts of phosphorus added were run at the same time in exactly the same way. Comparison of the colors obtained after various time intervals led to the conclusion that about 8 γ of phosphorus was present in each of our 30 mg. samples; that is, about 0.6 atom of phosphorus per molecule. Since hemoglobin *a* is more acidic than hemoglobin *b*,[9] it is clear that the phosphate content cannot be the cause of the difference.

Optical Rotation.—It is well known that denaturation of a protein often causes a significant shift in its isoelectric point. Hence hemoglobin *b* might represent some kind of denatured normal hemoglobin. We were, therefore, curious to compare the specific optical rotary power of the two substances. In many cases this property is rather sensitive to denaturation, especially when this process brings about a change in the status of the sulfur-containing amino acids. A Schmidt and Haensch polarimeter was used that allows measurements with an accuracy of about 0.01°. Optical rotation was measured in 20-cm. tubes containing 1% solution of normal adult, sickle cell trait, and fetal carbonmonoxyhemoglobin, in 0.01 *M* disodium hydrogen phosphate solution. The specific rotations found, using the red light obtained from a cadmium lamp by use of a red filter, were −23.5° for the normal, −24° for the fetal, and −23° for the sickle cell trait. Investigations with more concentrated solutions at other wave-lengths and with the concentrated solutions of the globins rather than with the whole pigments eventually might reveal significant slight differences. However, for our problem at the moment, the results are definite to the extent that they provide no positive indication that hemoglobin *b* is a denaturation product of normal hemoglobin.

Separation of Hemes from Globin.—The preparation of globin from hemoglobin deserves some comment; this process is of primary importance also in the dicussion of the electrophoretic measurements in a following paper.[4] The splitting of the carbonmonoxyhemoglobin into the porphyrin derivative and the globin was effected by acid acetone;[13] the process occurred more difficultly with hemoglobin *b* than with normal hemoglobin. This is of particular interest as it points to a difference between the two molecules in the region where the hemes are attacked. Such a difference was postulated by Pauling, Itano, Singer, and Wells[1] already in 1949 when they put forward a hypothesis on the mechanism of sickling in cells containing an appreciable amount of hemoglobin *b*.

The almost classical question of whether the splitting off of the heme from the globin part of the molecule is essentially connected with denaturation has been discussed over and over again. It is clear that certain changes take place in the protein when the heme is removed. However, it is our opinion, based on electrophoretic and other evidence, that if the splitting of carbonmonoxyhemoglobin is effected with great caution, a product can be obtained that still has the specific configurational features of the protein present originally in the hemoglobin molecule.[14]

Care should be taken to avoid the presence of oxygen during the reaction with dilute hydrochloric acid and acetone by working in a carbon monoxide atmosphere. Of special importance is the maintenance of a low temperature (0°C. or lower) throughout the preparation and the handling of the globin. Before electrophoretic or other measurements were performed with the globin, the solution of the hydrochloric acid salt of the globin in water was dialyzed for at least 24 hours at 4°C. against the buffer to be used. The buffer usually had a pH near the isoelectric point of the globin. During dialysis precipitation occurs of "irreversibly denatured" globin, the weight of which amounts to not more than 10% of the total in case the necessary precautions have been taken during the preparation. One has to be very careful with solutions of the globin even when the pH is near the isoelectric point and the molecules carry only a small charge. Keeping such solutions at 10 to 15°C. for a short period is sufficient to accelerate denaturation to such a degree that flocculation becomes visible. Addition of urea or of guanidinium chloride to a globin solution in concentrations of 5 to 10 *M* and subsequent removal of these reagents by dialysis in the cold leaves the protein strongly denatured, as becomes apparent from its insolubility at the isoelectric point. The globin from fetal hemoglobin proved to be especially prone to denaturation.

Terminal Amino Acid Residues.—A valuable method to check possible differences in chemical composition and structure between two proteins is the qualitative and quantitative determination of the terminal amino acid residues of the peptide chains of the protein molecules. For our

purpose we adopted the method of tagging the free amino end groups with 2,4-dinitrofluorobenzene, as has been worked out by F. Sanger.[10, 11] The separation and identification of the DNP amino acids obtained after hydrolysis was done by chromatography on columns of silicic acid and celite.[12]

Three pairs of determinations were made. In the first, the globins prepared from hemoglobins *a* and *b* were compared. One per cent solutions of the globins from hemoglobin *a* and from sickle cell trait hemoglobin ("*ab*") in 0.01 *M* disodium hydrogen phosphate were examined electrophoretically[4] before use, in order to establish the homogeneity of the sample in the one case and the presence of normal and abnormal globin in about equal amounts in the second case. The concentrations were estimated by measuring the absorption spectra in the ultraviolet region. Then 7.7 ml. of these solutions was treated with 0.1 ml. of dinitrofluorobenzene and kept in slow movement at 4°C. by mechanical rotation for 24 hours. Partial flocculation occurred in the course of time.[15] To the reaction mixture enough conc. hydrochloric acid was added to obtain a 6 *N* acid solution. Hydrolysis was effected by boiling under reflux for 20 hours. The terminal DNP-amino acids were extracted exhaustively with ether, and the combined ether layers were washed several times with 10^{-3} *N* hydrochloric acid and then evaporated to dryness *in vacuo*. Then a separation into groups was effected by chromatography.[12] Two main zones were observed in each case. The first ones in group III of the system[12] were identified as consisting mainly of dinitroaniline. The other, faster moving zones were purified and identified[12] as DNP-valine. For normal globin this result was to be expected on the basis of the analysis made by Porter and Sanger.[11] The zones were eluted with a mixture of alcohol and ether (1:4 by volume); the solutions were evaporated, and the DNP-valine was dissolved in glacial acetic acid. Measurement of the absorption spectra with the aid of a Beckman D.U. Spectrophotometer made it possible to calculate the amount of DNP-valine; $\epsilon_{max.}$ was assumed to be 1.6×10^4.

We found 2.04 μmoles of DNP-valine starting from 1.2 μmoles of normal globin and 1.87 μmoles of DNP-valine from 1.1 μmoles of sicklemic globin. Evidently there is no difference between the two proteins with respect to the amino end groups. Consideration of the losses due to destruction during hydrolysis (25%) and during isolation by chromatography and elution (10 to 15% for each chromatographic purification) leads to the value 4 as the number of terminal valine residues in each cases. There are indications in later experiments that the method of dinitrophenylation used did not effect a complete substitution of the amino groups, and the absolute value is hence unreliable; nevertheless the identity of the results obtained with normal and with sicklemic globin is important.

It was considered advisable to compare the blood pigments also by determination of terminal amino acids starting with the original hemoglobins.

The reaction with dinitrofluorobenzene was repeated with solutions of 250 mg. of normal and sickle cell anemia carbonmonoxyhemoglobin (the latter containing about 18% of fetal hemoglobin) in 7 ml. water to which 0.1 g. sodium bicarbonate had been added. After the reaction had gone on for 48 hours at 4°C. (precipitation occurred in the course of time) the substituted globins were set free and isolated by treatment of the reaction mixture with dilute hydrochloric acid and acetone. The preicpitates were washed with acetone until the washing liquid became colorless, and dried *in vacuo* over calcium chloride and potassium hydroxide (weight of the reaction products about 280 mg.). One hundred-fifty-milligram portions of the dinitrophenylated globins were hydrolyzed and analyzed in the usual way.

This experiment gave 25% more DNP-valine from the sickle cell anemia hemoglobin than from normal hemoglobin. The amounts could be estimated to correspond to 5 and 4 residues, respectively, per hemoglobin molecule. However, the dinitrophenylated globins were dissolved in dilute alkali and the ultraviolet absorption spectrum was measured; it was found that the extinction between 300 and 400 mμ, which must be ascribed to the whole of the dinitrophenyl groups in the proteins, and not only to those attached to the terminal valine residues, was also higher (by about 30%) with the sickle cell anemia preparation than with the normal material. Accordingly we conclude that the reaction with dinitrofluorobenzene proceeded somewhat more rapidly with hemoglobin *b* than with hemoglobin *a*.

An effort was made to carry out a complete dinitrophenylation. The reaction was carried out first by rotating 0.2 g. hemoglobin, 0.1 g. sodium bicarbonate, 0.1 ml. dinitrofluorobenzene, and 10 ml. water at 4°C. for 24 hours. Then 5 ml. of ethanol was added and the reaction was continued at 4°C. for 20 hours. Finally, the reaction mixture was brought to room temperature and mechanical rotation was continued for another 5 hours. The DNP-globins isolated were intensely yellow, suggesting the dinitrophenylation had proceeded well. From the hemoglobins *a* and *b* treated in this way the same amount of DNP-valine was isolated,[16] corresponding to about 5 terminal valine residues per hemoglobin molecule in each case.

Conclusion.—The evidence obtained in this investigation thus indicates a great similarity of the chemical composition of the protein of normal adult human hemoglobin and sickle cell anemia hemoglobin.

Acknowledgments.—The author feels indebted to Professor L. Pauling for his suggestions about research in this field and for the interest during the course of the investigations. He wishes to express his gratitude to Professors R. B. Corey and A. J. Haagen-Smit for their kind help during his work at the California Institute of Technology. Dr. W. A. Schroeder and Dr. F. Charlotte Green inaugurated the author into the chromatographic and other analytical methods worked out by their research group; they facilitated the investigations greatly by their assistance. Dr. H. A. Itano

kindly introduced the author into the special features of hemoglobin chemistry (see also the following publication). The author wishes to express his thanks to Dr. L. Zechmeister for his kind help in facilitating the use of the polarimeter and to Dr. A. Rich for his cooperation in the measurements with fetal hemoglobin.

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* Present address, Laboratory of Organic Chemistry, University of Leiden. The investigations dealt with in this and the following paper were carried out during a stay of the author in the United States sponsored by the Rockefeller Foundation (New York) and the U. S. Educational Foundation (the Hague).

† Contribution No. 1757.

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[7] Drabkin, D. L., *J. Biol. Chem.*, **164,** 703 (1946). We did not use the 1.2% NaCl + 0.0025 *M* $AlCl_3$ solution for the final washings, as recommended by this author, but washed the cells several times with 0.9% saline. Precipitation of the hemoglobin was effected by dialysis against conc. ammonium sulfate solutions.

[8] King, E. J., *Microanalysis in Medical Biochemistry, 2nd ed.*, New York, Grune and Stratton (1951).

[9] Following a proposal of H. A. Itano (these PROCEEDINGS, **37,** 775–784 (1951)) we shall, for brevity, designate normal adult hemoglobin as hemoglobin *a* and sickle cell anemia hemoglobin as hemoglobin *b*.

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[13] Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, **12,** 469 (1930).

[14] Spectrophotometric studies (Jope, E. M., Jope, H. M., and O'Brien, J. R. P., *Nature*, **164,** 622 (1949)) also point in this direction.

[15] Sorm, F., Korbl, J., and Matousek, L., *Coll. of Czechoslovak Chemical Communication*, **15,** 295 (1950).

[16] We got an indication of the presence of a small amount of DNP-methionine in the product from sickle cell anemia hemoglobin; more detailed investigations are needed to get definite information on this point.

ELECTROPHORETIC STUDIES OF GLOBINS PREPARED FROM NORMAL ADULT AND SICKLE CELL HEMOGLOBINS*

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Communicated by Linus Pauling, December 31, 1952

When the electrophoretic difference between normal adult hemoglobin and sickle cell hemoglobin was first detected, investigations were undertaken to determine whether the basis of this difference resides in the heme or the globin portions of these molecules. Dimethyl esters of the protoporphyrins were prepared from the hemes of each of these hemoglobins. X-ray diffraction photographs indicated that these protoporphyrin esters are probably identical.[1] Denatured globins were prepared from the hemoglobins of normal individuals and individuals with sickle cell trait and sickle cell anemia. Electrophoretic analyses of these denatured globins in acidic buffers failed to disclose any significant differences in composition or mobility.[2] The present study was undertaken in order to compare the electrophoretic properties of the native globins, which, unlike the denatured globins, are soluble in their isoelectric region.

Experimental Methods and Results.—The separation of heme from globin was accomplished by use of the acid acetone method of Anson and Mirsky.[3] The globin thus prepared was dialyzed against 0.01 *M* Na_2HPO_4 to effect reversal of the denaturation caused by the acid acetone. The loss in insoluble protein approximated 10%. The native globins were then examined in the Tiselius apparatus in 0.01 *M* Na_2HPO_4 and in cacodylate buffer of pH 7.0 at potential gradients of 16.6 and 5.0 volts per centimeter, respectively. The cacodylate buffer was 0.08 *M* in NaCl, and its ionic strength was 0.1.

Although 0.01 *M* Na_2HPO_4 is a useful solution in which to detect small mobility differences between components in mixtures of hemoglobins,[4] its buffering capacity is poor. Hence, reproducible values of absolute mobilities are difficult to obtain in this solution, and the results are best discussed in terms of differences in mobilities. Figure 1, (*a*) and (*b*), illustrates the scanning diagrams obtained following electrophoresis of native globins prepared from normal adult and sickle cell trait hemoglobins. These globins were dialyzed simultaneously against the same solution of 0.01 *M* Na_2HPO_4 and were examined under identical conditions. Only one major component is evident in the preparation of normal adult globin. In the sickle cell trait globin two components are present, one of which has the same mobility as the normal adult globin. The absolute mobility of the other component in sickle cell trait globin is lower than that of the normal adult

globin, by 0.6×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}. Each of these globins migrated as a negative ion in this system.

Figure 1, (*c*) and (*d*), shows two different globin preparations examined in 0.01 *M* Na_2HPO_4 on different days. A large fraction of the globin prepared from sickle cell anemia globin appears to be homogeneous. The small component of lower mobility may represent a denaturation product. The native globin prepared from a mixture containing nor mal adult and sickle cell anemia hemoglobins in approximately equal amounts shows considerable heterogeneity. The mobility difference between the two major peaks is the same as that between the two components in sickle cell trait globin. The sickle cell anemia hemoglobin in these experiments contained less than 5% fetal hemoglobin.

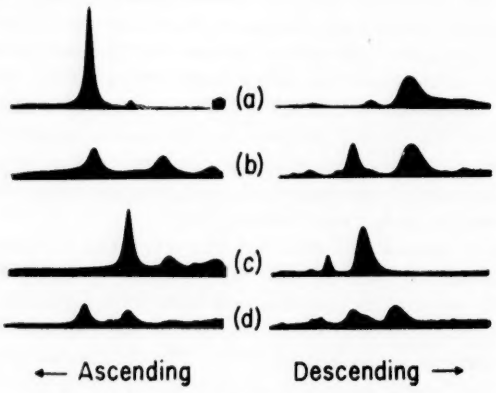

FIGURE 1

Longsworth-scanning diagrams of globins prepared from human hemoglobin. (*a*) Normal adult globin, 22,860 sec.; (*b*) sickle cell trait globin, 22,860 sec.; (*c*) sickle cell anemia globin, 14,400 sec.; (*d*) globin prepared from a mixture of normal adult and sickle cell anemia hemoglobins, 14,400 sec. Electrophoresis in 0.01 *M* Na_2HPO_4 at 16.6 volts per centimeter and 1.5° C.

On the acid side of the isoelectric point, in cacodylate buffer of pH 7.0, the mobilities were 2.6×10^{-5} and 3.4×10^{-5} cm.^{2} sec.$^{-1}$ volt,$^{-1}$ respectively, for normal adult and sickle cell anemia globins.

Normal and sickle cell anemia globins were treated with 4 *M* guanidinium chloride for 1 hour at 4°C. Following removal of the guanidinium chloride by dialysis, the globins were no longer soluble in 0.01 *M* Na_2HPO_4. Examination in the cacodylate buffer of pH 7.0 revealed a marked increase in heterogeneity. The mean mobilities of the denatured specimens in this buffer were nearly the same, 2.8×10^{-5} and 2.9×10^{-5} cm.^{2} sec.$^{-1}$ volt,$^{-1}$ respectively, for normal adult and sickle cell anemia globins.

Discussion.—The presence of only one major component in the native globins prepared from normal adult and sickle cell anemia hemoglobins but of two in the native globins prepared from sickle cell trait hemoglobin and a mixture containing both normal adult and sickle cell anemia hemoglobins suggests that normal adult and sickle cell globins differ in their globin portions. The mobility relationship between the globins is similar to that between the corresponding hemoglobins in that the normal protein has a greater mobility on the alkaline side of the isoelectric point. The mobility difference between the native globins is slightly higher than that observed between the hemoglobins.[1]

Since the amino acid compositions of normal adult and sickle cell hemoglobins are nearly the same,[5] and since the acid denatured globins do not differ significantly in their electrophoretic behavior, it has been suggested that the two hemoglobins might have identical polypeptide chains, and that their electrophoretic difference results from a difference in the way in which these identical chains are folded in the native molecule.[6] It was found in the present investigation that the dialysis of acid-acetone treated globin against an alkaline buffer resulted in soluble globins which corresponded in composition and relative mobility to the hemoglobins and hemoglobin mixtures from which they were prepared. Treatment of the soluble globins with guanidinium chloride resulted in heterogeneous mixtures which did not differ significantly from each other in their mean mobilities. These observations suggest that, if the polypeptide chains are identical, the denaturation which takes place when heme is removed by treatment of hemoglobin with acid acetone does not completely unfold the chains. Each globin retains a sufficient part of its specific configuration to enable it to return to its native configuration upon treatment with a weakly alkaline phosphate solution. The denaturation due to guanidinium chloride apparently was not reversed upon removal of the denaturing agent.

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§ Contribution No. 1759.

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THE EFFECT OF AMMONIA ON MORPHOGENESIS IN THE ACRASIEAE*

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Communicated by F. W. Went, November 10, 1952

The peculiar separation of mass increase and morphogenesis in the *Acrasieae* has led to renewed interest by several workers, among them Raper,[1, 2] Bonner,[3] Gregg,[4] and Sussman,[5] all of whom have taken advantage of this fact. The remarkable life history is well given by Raper[6] and by Bonner.[7] The present paper is the initial report of a program of studies on the influence of physicochemical factors in the morphogenesis of these organisms.

The *Acrasieae* described by Olive[8] in the most extensive monograph of the group show a well-graded series of increasing complexity as pointed out by Raper[6] from the almost chance aggregation of cysts in *Sappinia* through sessile and stalked fruits in *Guttulina* and *Guttulinopsis* in which stalk and spore cells are indistinctly differentiated to the complex differentiation of stalk and spore mass in *Dictyostelium* and a regularly branching arrangement in *Polysphondylium*. It would therefore be worth while to get a range of these forms in culture for the study of the factors involved in increasing morphogenetic complexity.

For ten years I have cultured soil samples as opportunity permitted in an attempt to obtain a series of forms. During the last two years a systematic selective culture of 79 soil samples from diverse habitats in Georgia, Florida, Massachusetts, and Michigan has yielded about as many strains of *Dictyostelium* and *Polysphondylium* species, but no member of the "lower *Acrasieae*." Raper (personal communication) with more extensive experience has confirmed this observation. On a few occasions what seemed to be sessile *Guttulinas* developed into *Polysphondylium* and *Dictyostelium* on transfer to laboratory media.

The occasional appearance of forms resembling macroscopically in every respect Olive's[8] illustrations of *Guttulina* and *Guttulinopsis*, especially in old cultures on a rich supply of bacteria, led to the possibility that these genera might be artifacts of culture.

Experimental Methods and Results.—Isolation and culture methods are presented in detail elsewhere,[9] but except where otherwise noted, spores were inoculated in the center of a plate prepared by spreading washed *Escherichia coli* uniformily on non-nutrient agar. Under proper conditions there is an expanding circle of proliferation followed by a graded series of morphogenetic stages from mature sorocarps in the center to pseudoplasmodium formation at the inner border of the expanding annulus of vegetative

amebae. Hence one can note the influence of external conditions on any stage.

To study the effect of humidity, plates[10] of *Dictyostelium discoideum* 14, *D. purpureum* 11, *Polysphondylium violaceum* 19, and *P. pallidum* 15 were placed under bell jars through which flowed streams of air at humidities ranging from 5 to 95%. While there was some arrest of development and the production of dwarf forms at the lower humidities, the sorocarps were essentially normal.

In a series of experiments with *Polysphondylium pallidum* 15 and *Dictyostelium mucoroides* 43, the organisms were grown in two-membered culture on *E. coli* on peptone-dextrose 2% agar slants buffered with KH_2PO_4–NaH_2PO_4 at pH 6, total phosphate M/75, in which the ratio of peptone and dextrose was varied. The results are shown in table 1.

TABLE 1

DEXTROSE, %	ORGANISM	PEPTONE, % 0.05	0.1	0.2	0.5	1.0	2.0
0.05	*Pol.*	+++	++	++	++	+*P*	–
	Dic.	++	+++	+++	++++	++++	++
0.1	*Pol.*	+++	++++	+	+	+*P*	–
	Dic.	+	++	+++	+++	++++	++*G*
0.2	*Pol.*	+++	++++	++++	+	+*P*	–
	Dic.	++	+++	++++	++++	+++*G*	+++*G*
0.5	*Pol.*	++	+++	+++	++*G*	++*G*	–
	Dic.	+	++	+++	++++	++++	+++
1.0	*Pol.*	+++	++	++	++*G*	+	+*P*
	Dic.	+	++	+++	++++	++++	+++
2.0	*Pol.*	+	+	+	+	+	+*P*
	Dic.	+++	+++	++++	++++	++++	+++*G*

To separate the pH factor from that of the effect of metabolites, five *Acrasieae* were selected to cover a wide range of types (*Dictyostelium purpureum* 11, *D. discoideum* 14, *D. mucoroides* 43, *Polysphondylium pallidum* 15, *and P. violaceum* 46). The agar was adjusted with phosphate and phosphate-citrate buffers to provide approximately 0.4 pH unit steps from pH 3.1 to pH 10.6 after autoclaving. *E. coli* was spread on these non-nutrient agars and the *Acrasieae* inoculated. After the experiments were ended the pH of each plate was again taken, and it was found that they varied from pH 3.2 to pH 8.9. The pH in these experiments was a complex function of the quantity of *coli*, the quantity of agar, and the efficiency of the buffer; control plates inoculated with *E. coli* but not with *Acrasieae* showing a similar drift from both the acid and alkaline range toward a slight alkalinity (i.e., plates in the range pH 7.8 – 8.0 showed little or no change).

On phosphate buffered agars, no *Acrasieae* showed growth at pH 3.1 (final pH of plates 3.2 – 3.4). *Polysphondylium pallidum* showed normal

growth and development at pH 3.4 (final pH 3.5) while *Dictyostelium mucoroides* first showed normal development at pH 3.8 (final pH 6.4) and *Polysphondylium violaceum* at pH 4.1 (final pH 6.6). Because of the lower buffering capacity of this latter range of mixtures the results are doubtful. Titration curves with $H_3PO_4 - KH_2PO_4$, and KH_2HPO_4 systems, $M/75$ total PO_4 in 2% agar, showed regions of highest buffering efficiency in the approximate ranges pH 2.7 – 3.7, pH 6.5 – 7.8, and between pH 10.4 and 10.9, though at this highest range the results are uncertain because of the limitations of the glass electrode. Buffering was practically nil between pH 4.1 and 5.6.

In a phosphate–citrate system, with better buffering throughout its range, *Dictyostelium mucoroides* showed normal development at pH 3.4 (final pH 4.4) while *Polysphondylium violaceum* showed a few normal sporangia at pH 4.2 (final pH 4.8) and completely normal development at pH 4.6 (final pH 5.5), confirming the acid sensitivity of these two strains. On the alkaline side all showed normal development at an initial pH of 10.6 which varied at the conclusion of the experiment from pH 8.9 to 8.5. This result was twice confirmed in other experiments and checked colorimetrically as well as with the glass electrode.

There was an appearance of *Guttulina*-like forms with *Polysphondylium pallidum* cultures and in fewer cases with *Dictyostelium mucoroides* with a final pH of 6.85 or above. *Polysphondylium violaceum* showed above a final pH of 8.5 an increased tendency toward the development of unbranched *Dictyostelium*-like fruits. The only correlations which could be made were that these forms tended to appear: (*a*) at the periphery (i.e., last part of the plate to be cleared of bacteria); (*b*) where the bacteria were thickest in irregularly coated plates; and (*c*) in occasional plates with pH higher than approximately 6.5, but without correlation with the degree of alkalinity, except for the *Dictyostelium*-like forms of *Polysphondylium Violaceum*.

Since the degree of alkalinity of the medium was not responsible for the aberrant morphogenetic expression, exploratory experiments were made with filtrates of *E. coli* cultures in peptone-dextrose and in peptone, adjusted to pH 6.0 and made into agar, then covered with heat-killed *E. coli*. When inoculated with pure cultures of *Polysphondylium pallidum* 15 these plates showed only plaques of amebae with peptone alone; *Guttulina*-forms with 1% peptone — 0.5% dextrose, and with 1% peptone — 1% dextrose; and normal development on control 1% peptone which had not been previously inoculated with *coli*, and on the pH 6.0 non-nutrient plates. Normal development occurred in mold-contaminated plates prepared from the culture filtrates, confirming Raper's [11] observations.

The evidence was strong that neither pH nor peptone *per se* was directly responsible for the development of *Guttulina*-like forms, and that some

metabolite formed in the absence or relative scarcity of dextrose by *E. coli* was the responsible factor for either suppression of morphogenesis or its aberrant expression. Ammonia seemed the most likely factor; rough quantitative Nesslerization of the broths indeed showed an inverse relation between the dextrose concentration and ammonia; the uninoculated peptone showing no trace of ammonia.

Therefore experiments were set up in which *E. coli* plates were inoculated with *Polysphondylium pallidum* 15 and 25, *P. violaceum* 19, and *Dictyostelium purpureum* 11, and inverted over vessels containing watch glasses in which various concentrations of ammonium chloride solution were placed and from which ammonia was generated by mixing with sodium hydroxide. A typical experiment was as follows: plates in duplicate; each over a watch glass containing 1 ml. *M*/10 NaOH, 1 ml. ammonium chloride solution of concentration indicated in table 2; solutions changed daily, experiment

TABLE 2

MOLAR CONCENTRATION OF AMMONIUM CHLORIDE (1 ML.) ADDED TO 1 MOLAR SODIUM HYDROXIDE (1 ML.)

ORGANISM AND STRAIN	0.2 *M*	0.1	0.05	0.025	0.0125	CONTROL
D. purpureum 11	–	*N, G, A**	*N*	*N*	*N*	*N*
P. pallidum 15	–	–	*A*	*G, A*	*N*	*N*
P. pallidum 25	–	–	*N, D, A*	*N*	*N*	*N*
P. violaceum 19	–	*G, S, T, D*	*N, G*	*N*	*N*	*N*

* *A* = ring of amebae or plaques; no essential difference is assumed between vegetative growth in the form of distinct plaques or of rings. *G* = *Guttulina*-like forms, i.e., sessile more or less spherical masses, *S* = minute stalked forms resembling *Guttulinopsis* with imperfectly differentiated stalk and sorus. *D* = *Dictyostelium*-like (unbranched) forms in the normally branched *Polysphondylium*, *T* = tapering form stalk ending in a single row of cells, perhaps recalling van Tieghem's[8] original Acrasis, not found since its discovery, while *N* = normal appearance for the species and – = complete inhibition of growth.

lasted four days at approximately 22°C., constant overhead illumination to prevent lateral extension of sporangia toward window. The results are summarized in table 2. It can be seen from this table that these four forms vary in sensitivity, *Polysphondylium pallidum* 15 being the most sensitive and *Dictyostelium purpureum* the least. There is also apparently a difference in sensitivity between strains 15 and 25 of *P. pallidum*, an impression confirmed in other experiments.

Several repetitions of this experiment under slightly varied conditions established the role of ammonia as the morphogenetic suppressor. In *Polysphondylium pallidum* 15 and 25, inoculation of *Guttulinas* into standard *coli* plates resulted in normal *Polysphondylium*.

In all succeeding work spot checks were made of plates and tubes in which *Guttulina*-forms developed. Ammonia could be shown by Nessler's

reagent in all such cases tested, but not in those with solely normal sporangia. *Coli* suspensions which had been kept for a week or more in the refrigerator showed a positive test for ammonia; fresh suspensions did not. The formation of *Guttulina*-like fruits could be obviated by any one or a combination of the following procedures: using fresh suspensions, using freshly prepared plates, inverting the plates over dishes containing acid (e.g., $M/20$ H_2SO_4) to trap the ammonia, using plates with a high agar/coli ratio to dilute the ammonia, and using pure cultures on heat-killed (70°, $^1/_2$ hour) *E. coli*, though contamination with molds is almost inevitable in the latter case.

That free ammonia and not the ammonium ion is the factor was indicated by placing filter papers moistened with $M/10$ ammonium chloride on coli plates. Normal sporangia not only developed around the filter papers but through them as well.

Confirmation was obtained by the development of normal sporangia of *Polysphondylium pallidum* and of *Dictyostelium purpureum* on *Coli* plates containing ammonium chloride at $M/33$ concentration over $M/20$ sulfuric acid to absorb any free ammonia produced. This represents a concentration of ammonium ion many times greater than the concentration of free ammonia necessary to produce inhibition of development or the production of abnormal forms.

Another factor in morphogenesis is carbon dioxide. In experiments in which plates were sealed over sodium hydroxide placed in Bray anaerobic culture dishes, there was no development. Replacement of the sodium hydroxide with sodium bicarbonate as the ammonia-generating alkali gave essentially the same results as shown in table 2. There was also no development in the controls over sodium hydroxide while those over acid showed vigorous growth and completely normal fructifications.

The carbon dioxide–ammonia relationships are still under study; besides the apparent necessity of carbon dioxide for any sort of development, there is evidence that an antagonism exists between these two chemicals. A detailed report is being prepared on the quantitative relationships.

Discussion and Conclusions.—The results given here support Raper's findings[11] in many respects. I, too, have found that the use of fermentable sugars allows the development of the *Acrasieae* and that a roughly 1:1 ratio of peptone : dextrose is optimal. In experiments not otherwise reported in detail my findings are in agreement with his,[2] that some species or strains can tolerate higher concentration of nutrients than others. In agreement with Raper I also found that accidental contamination with molds allowed the morphogenetic expression of various *Acrasieae* to take place where it was otherwise suppressed. In high peptone: dextrose ratios Raper observed *Guttulina*- and *Guttulinopsis*-like forms, while Singh[12] in a study of bacterial-acrasian relationships remarked the similarity between his "abnormal"

forms and the genera *Guttulina* and *Guttulinopsis* as described by Olive.[8] Singh states: "As the genera *Guttulinopsis* and *Guttulina* were described before the cultural characteristics of the amoeboid protists included in the *Acrasieae* were properly understood, it will be necessary to study these genera, if they are rediscovered, under controlled cultural conditions before they can be considered valid." Such a study is being made at present; and comparison of Olive's original material macroscopically and microscopically with the "abnormal forms" of *Polysphondylium* indicates no significant differences as yet.

Inspection of table 1 shows that *Polysphondylium pallidum* is more sensitive to higher concentrations of *E. coli* metabolites in general than *Dictyostelium mucoroides*. This confirms Raper's findings.[2]

The interpretation of these results differs from that of Raper.[11] While he showed that for *Dictyostelium discoideum* high pH was inimical to the full development of this Acrasian and expressed the belief that the high pH was caused by the formation of ammonia, the extended experiments reported here indicate that free ammonia is the primary morphogenetic suppressant and alkalinity is not responsible. Singh (*loc.cit.*) obtained similar results and expressed a belief that the abnormalities were caused by some metabolite.

It is interesting to note (Stephenson,[13]) that in the metabolism of amino acids by bacteria, decarboxylation is predominant under acid conditions or in the presence of fermentable carbohydrates, while deamination is predominant under alkaline conditions and is inhibited by fermentable carbohydrates.

Recently Sussman [14] announced the finding of mutants in *Dictyostelium discoideum* and in *D. purpureum* which from his photographs resembled the forms obtained in this laboratory in varying the carbon dioxide and ammonia relationships. His organisms were grown in two-membered culture with *Aerobacter aerogenes* on a nutrient agar, and some of his "aggregateless" forms (i.e., plaques only) formed normal sporangia when the amebae were washed off the nutrient and placed on a fresh plate. From these facts and the data exemplified in table 1, it may be inferred that at least in some cases the mutation represents increased sensitivity to ammonia.

Finally the question of abnormality comes up. We perhaps unconsciously assume the criterion of normality to be ability to reproduce. This is certainly the criterion of the microbiologist. By this definition the "*Guttulinas*" found in these experiments are "normal."

Summary.—Ammonia is found to simplify or suppress morphogenetic expression in certain *Acrasieae*, either inhibiting growth completely, allowing the development of plaques of vegetative amebae in lower concentrations, or when still more dilute, allowing the formation of simple fructifications resembling the presumptive genera, *Guttulina* and *Guttulinopsis*. Normal

development, e.g., of *Polysphondylium* occurs when ammonia is present in traces or is undetectable.

Acknowledgment.—I am greatly indebted to Dr. Kenneth B. Raper for checking the identifications of these *Acrasieae* and for returning subcultures in lyophilized form.

* Supported by a Grant-in-aid from the American Cancer Society upon recommendation of the Committee on Growth of the National Research Council

[1] Raper, K. B., *J. Elisha Mitchell Sci. Soc.*, **56**, 241–282 (1940).

[2] Raper, K. B., *Quart. Rev. Biol.*, **26**, 169–190 (1951).

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[5] Sussman, M., *Ibid.*, **118**, 407–418 (1951).

[6] Raper, K. B., *Am. J. Bot.*, **27**, 436–448 (1940).

[7] Bonner, J. T., *Morphogenesis*, Princeton University Press, Princeton, 1952.

[8] Olive, E. W., *Proc. Boston Soc. Nat. Hist.*, **30**, 451–513 (1902).

[9] Cohen, A. L., *Ann. N. Y. Acad. Sci.* (in press).

[10] The number following the species refer to my Acr. series of isolates. Strain Acr. 43 is called *Dictyostelium mucoroides* in the paper but Raper (personal communication) states the following: "*Dictyostelium mucoroides*, probably. Some sorophores up to 3.5 cm. Spores elliptical, but relatively broad, measuring 6–7μ × 4.5–5μ."

[11] Raper, K. B., *J. Agr. Res.*, **58**, 157–198 (1939).

[12] Singh, B. N., *J. Gen. Microbiol.*, **1**, 11–21 (1947).

[13] Stephenson, M., *Bacterial Metabolism*, 2nd ed., Longmans, London, 1949, p. 134.

[14] Sussman, M., Conference on Growth of Protozoa, N. Y. Acad. Sci., October 27, 1952 (to be printed in *Ann. N. Y. Acad. Sci.*).

BIOMETRIC EVIDENCE OF NATURAL SELECTION IN ASCLEPIAS TUBEROSA

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Asclepias tuberosa, commonly known as butterflyweed and orange milkweed, consists of four subspecies separable primarily on leaf shape. The subspecies are isoploid and cross pollinated, and hybridize introgressively at the juncture of their ranges.[1]

Of particular interest are subspecies *interior* and *terminalis*[2] which are sympatric and occupy a vast area from the Great Lakes to northern Mexico. *Interior* bears leaves which typically are broadly cordate or lobed at the base, while the leaves of *terminalis* are cuneate or tapered at the base. Although one occasionally may find plants with leaves characteristic

of one subspecies well within the range of the other, the most deeply cordate leaves are found in the greatest concentration in the central Ozarks of southeastern Missouri. Outward from this center leaves become on the whole less cordate and more cuneate progressively to the periphery of the species range on all sides.

This peripheral effect is known in other plants and animals. Study of its nature in *Asclepias tuberosa* is facilitated by a method of measuring the shape of the leaf base which I devised several years ago.[1] This method, which is remarkably sensitive and accurate, would assign an angular value (called $\angle B$) of more than 90° to a cordate leaf base, of less than 90° to a cuneate leaf base, and of exactly 90° to a leaf base exactly perpendicular to its longitudinal axis, which the descriptive taxonomist would call truncate.

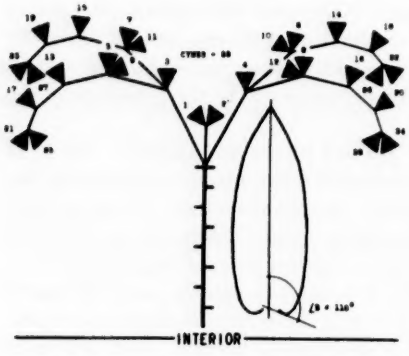
In my first study of leaf variation in *Asclepias tuberosa*, after measuring several thousand leaves from as many herbarium specimens within the range of ssp. *interior* and *terminalis*, I demonstrated quantitatively the centrifugal decrease of $\angle B$ by assigning the various $\angle B$ values obtained to appropriate circles inscribed upon a map and concentric from an arbitrary center in the Missouri Ozarks. Lastly, to determine what ecological and latitudinal factors might be involved, the artificial populations from the concentric circles were divided to supply opposed series of concentric arcs from the point of origin in the Ozarks to the northeast and to the southwest, respectively. The two curves of $\angle B$ values (lower panel of Fig. 1), which I have not attempted to smooth, were found to be nearly identical in spite of the widely differing climatic conditions encountered—and particularly so in view of the limited samples available.

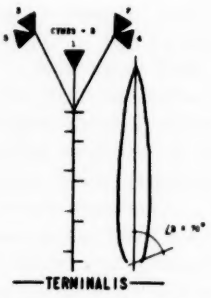
I have had cultures of both subspecies under observation in my garden for several years and the genetic constancy of the phenotypes has been confirmed. Under cultivation it is impossible for me to distinguish plants of ssp. *terminalis* from such remote areas as central Minnesota and southwestern Texas, as it is in the herbarium, whatever subtile ecotypic responses may be involved.

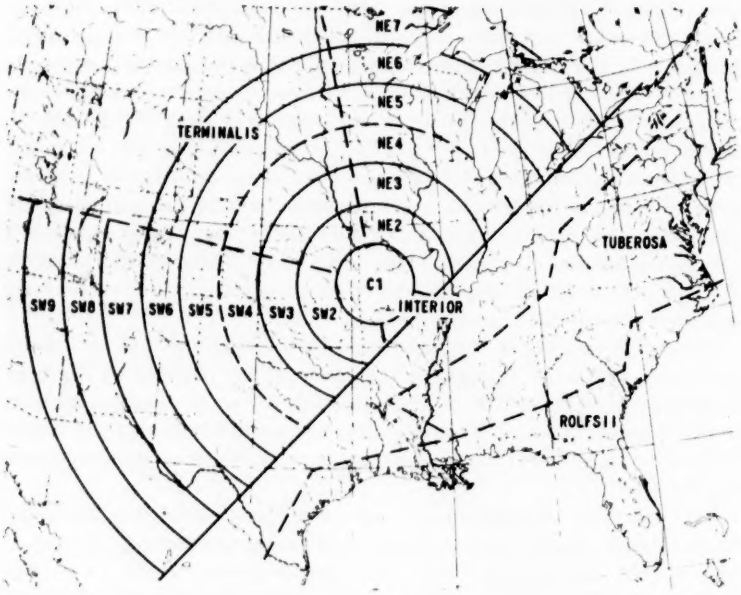
That two easily distinguishable populations such as ssp. *terminalis* and *interior* should assume their strikingly regular concentric ranges independently is quite unthinkable. It is even more so to consider the central ssp. *interior* to be the spontaneous product of hybridization from the periphery, which has been suggested to me.

The most reasonable interpretation, in my opinion, is to view ssp. *terminalis* as an ancient, ecologically very tolerant population, and ssp. *interior* as a relatively recent genetic innovation within it probably dating much more recently than the retreat of the glaciers, to judge from the remarkably even phenocontours both to northeast and to southwest.

It long has seemed to me that the peculiar concentric distributions of ssp. *interior* and *terminalis* provide a study in natural selection in which the


INTERIOR


TERMINALIS


NE7
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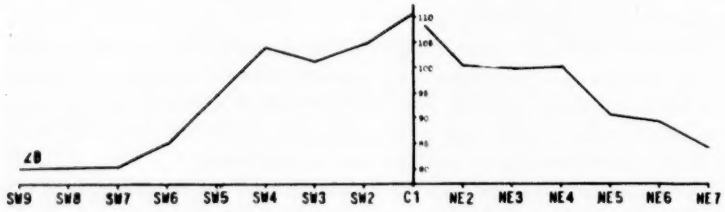
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presumably older *terminalis* is being supplanted by the younger, more aggressive ssp. *interior* through introgressive hybridization and some selective superiority transmitted by the latter to its heterozygous progenies. During the past summer opportunity to continue the problem in this light was given me by grants from the BACHE FUND of the NATIONAL ACADEMY OF SCIENCES and the Permanent Science Fund of the American Academy of Arts and Sciences.

To this end, an automobile trip was taken from about the middle of Circle 1 in the central Ozarks (middle panel, Fig. 1) due north to the limits of Arc NE 5, about one hundred miles north of Minneapolis. Thirty colonies of *Asclepias tuberosa* were encountered, and a random flowering stem taken from each plant. The colonies averaged about 25 plants. Although in previous studies only leaves were measured, in this case several additional measurements and counts were made.

In sum, it was found that the total number of flowers produced by a plant bearing cordate leaves (i.e., ssp. *interior*) usually is significantly greater than the number produced by plants with cuneate leaves (i.e., ssp. *terminalis*). Flowers in *Asclepias* are borne in small individual clusters called cymes, and do not open for pollinating agents all at one time, but progressively. The number of flowers borne in the cymes does not differ significantly in these two subspecies of *Asclepias tuberosa*, but a typical stem of ssp. *interior* produces more cymes than does one of ssp. *terminalis*. This accounts for the differing totals of flowers produced, and consequently the number of cymes has been used as the unit of measure in this study. Furthermore, like the flowers, the cymes do not mature at one time, but in succession.

It thus follows that the larger number of cymes produced by a typical plant of ssp. *interior* not only produces more pollen but also over a correspondingly longer period than the smaller number of cymes characteristic of a plant of ssp. *terminalis*. One might expect that ssp. *interior* would be a more effective pollinator than ssp. *terminalis* not only in initial hybridization but exponentially so in back crosses. I believe it reasonable to assume that this character of ssp. *interior*, together incidentally with a probably greater seed production as well, is of very positive selective value, and that the character of the cordate leaves, among others, is merely associated with it in its conquest of the older ssp. *terminalis*.

Space does not permit extensive report of data collected during the past summer; a more detailed account will be given elsewhere. However, very interesting effects are obtained by projecting the colonial means of the thirty

FIGURE 1

Upper panel: *Asclepias tuberosa* sspp. *interior* and *terminalis* contrasted. Middle panel: method of studying internal differentiation in *A. tuberosa*. Lower panel: profile of genoclines in sspp. *interior* and *terminalis*. Explanation in the text.

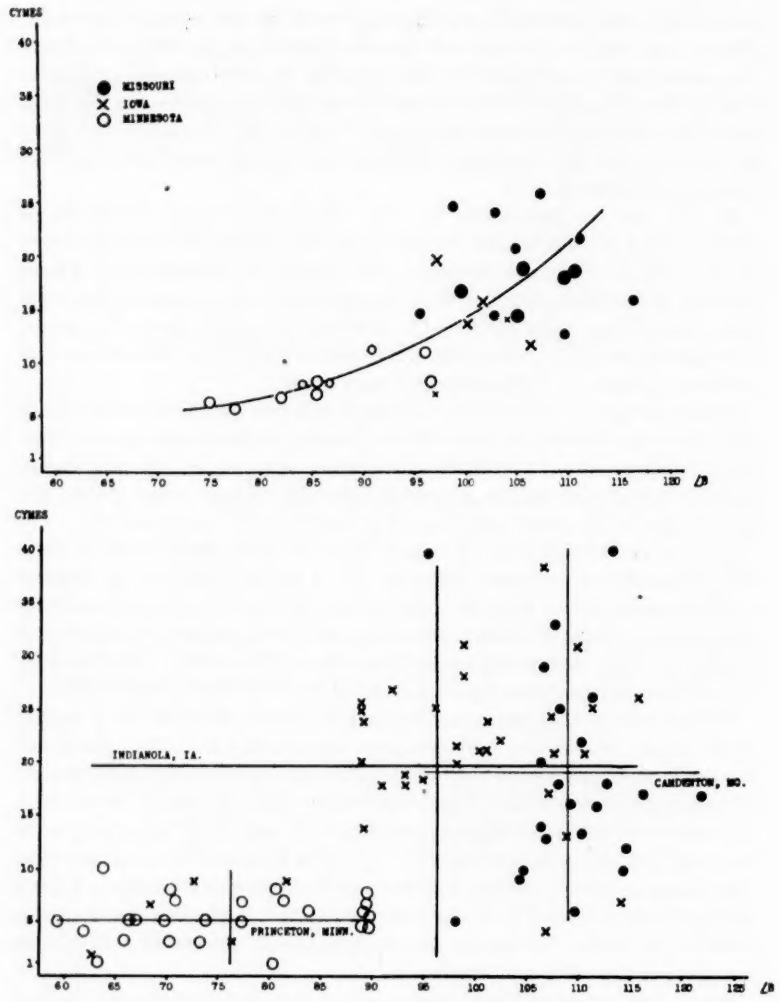

FIGURE 2

Upper panel: colonial means of thirty colonies of *Asclepias tuberosa* sspp. *interior* and *terminalis*. Lower panel: individual plants of three colonies of the above. Explanation in the text.

colonies sampled in Missouri, Iowa, and Minnesota upon a grid for cyme number and $\angle B$ (upper panel of Fig. 2). Particular attention should be called to the high variability of the Missouri colonies and the relatively low variability, particularly for cyme number, of the Minnesota colonies as would be expected from Vavilov's[3] theory of the centers of origin of cultivated plant species if our hypothesis were correct. It will also be noticed that the recombination pattern assumes the properties of an exponential curve.

In judging whether introgressive hybridization is the implement of natural selection in *Asclepias tuberosa*, the lower scatter diagram of figure 2 is helpful. Here the individual plants of three colonies are plotted for cyme number and $\angle B$. A number of interesting and suggestive properties are seen. But of immediate interest are the distinctive and relatively uncorrelated distributions of the colonies from Minnesota and Missouri and the greater variability and higher correlation of the Iowa colony, wholly indicative of heterozygosity for these characters, I believe. Although both characters apparently are multifactorial, notice the apparent segregation of the Iowa colony into blocks simulating a monofactorial F_2.

In my opinion the pre-eminent evolutionary significance of introgressive hybridization in both plants and animals is as a vehicle for natural selection. Indeed, particularly if systemic mutations are dismissed, if might be considered the principal vehicle in sexually reproducing organisms. No other mechanism could be more natural nor more subtile for the statistical transformation of populations essential to the Darwinian theory. I consider the process of hybridization under selection pressure to be so fundamental to organic evolution that, as an inveterate descriptive taxonomist, I am giving it a name: sabinism. This will recall the violent fusion of populations which formed Rome, particularly the early military and genetic conquest of the Romans over their neighbors the Sabines, and familiar to most of us through the painting of Louis David.

[1] Woodson, R. E., Jr., *Ann. Missouri Bot. Gard.*, **34**, 353–432 (1947).

[2] *Asclepias tuberosa* ssp. *terminalis* Woodson, subspec. nov.—Subspecei *interiori* affinis et cum ea vulgo conjugalis, foliis lanceolatis basi saepius cuneatis vel truncatis rarius minus cordatis inflorescentia minus floribunda differt. Typus: *L. S. Ehlers 13954* in herb.—Missouri Botanical Garden (Burt Lake, Cheboygan Co., Michigan).

[3] Vavilov, N. I., *Studies on the Origin of Cultivated Plants*. Leningrad, 1926.

A STUDY OF LIGNIN FORMATION BASED ON THE OXIDATION OF NATIVE AND ENZYMATICALLY LIBERATED LIGNINS*

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Communicated by W. J. Robbins, December 4, 1952

The application of oxidation reactions in the elucidation of the structure of natural products has had wide usefulness and its value is well established. Some oxidizing agents employed in the degradation of lignin are potassium permanganate,[1] metallic oxides,[2] hydrogen peroxide,[3] nitric acid,[4] and nitrobenzene in alkali.[5a,b] By far the mildest of these is the last mentioned. The degradation products usually obtained by this method are vanillin from softwood lignins[6] and vanillin and syringaldehyde from the hardwood[7] and annual plant lignins.[7] Recently, paper partition chromatography has been applied for the isolation and identification of the aldehydes obtained by nitrobenzene–alkali oxidation of lignin materials.[8] Using these two methods has led to the identification of *p*-hydroxybenzaldehyde as a degradation product of the native and enzymatically liberated lignins from bagasse.[9] Since a preparation devoid of nitrogenous materials or other non-lignin impurities served as a substrate, the presence of a non-methoxylated aromatic building stone in this annual plant lignin is firmly established. Thus, it was felt that a logical extension to these studies would be the oxidation of the native and enzymatically liberated lignins from representative softwoods and hardwoods.

TABLE 1

Oxidation Products Derived from Native and Enzymatically Liberated Lignin

Lignin	*p*-Hydroxybenzaldehyde, %	Vanillin, %	Syringaldehyde, %
White Scots Pine N. L.[a]	3.5	19.5	..
White Scots Pine E. L.[b]	2.4	18.7	..
Oak N. L.	...	21.3	4.0
Oak E. L.[c]	...	20.9	3.6
Birch N. L.	>0.5	18.6	1.7
Birch E. L.[c]	>0.5	19.1	2.0
Maple N. L.	...	17.2	4.5
Maple E. L.[c]	...	16.9	4.2
Bagasse N. L.	9.8	17.8	13.3
Bagasse E. L.[d]	10.2	17.3	12.9
"Kiri" N. L.	...	20.8	11.2

[a] Native lignin.
[b] Lignin liberated by *Lentinus lepideus*.
[c] Lignin liberated by *Daedalea quercina*.
[d] Lignin liberated by *Poria vaillantii*.

Experimental.—The native lignins used in this study were isolated according to the method outlined previously[10] and the enzymatically liberated lignins were obtained by our procedure.[11a,b] The oxidation of the lignins and the quantitative determination of the degradation products is based on a previously described method.[8]

The phenylhydrazone derivatives of the white Scots pine native lignin and its diazomethane methylated derivative were prepared as usual.

A Beckman quartz spectrophotometer was employed in determining the ultraviolet absorption curves. The solutions were prepared by dissolving 3 to 4 mg. of lignin per 100 ml. of solvent (90 parts of purified dioxane to 10 parts of distilled water).

Results and Discussion.—The results of the oxidation are recorded in table 1. It is significant that the white Scots pine lignins (native and enzymatically liberated) gave appreciable amounts of *p*-hydroxybenzaldehyde as a degradation product.[12] Of the hardwood lignins investigated only birch lignin yielded this aldehyde and then in only very small amounts; i.e., less than 0.5%.

A reinvestigation[11b] of the oak and birch lignin degradation products with the aim of establishing the presence or absence of *p*-hydroxybenzaldehyde led to the identification of small amounts of syringaldehyde. These lignins were then treated according to the Mäule reaction.[13] Each gave a cerise color test although that of the birch lignin was very weak. The oak and birch lignins were also oxidized with nitrobenzene in alkali in the presence of a known amount of added pure syringaldehyde. On spraying the paper chromatogram with 2,4-dinitrophenylhydrazine reagent, the syringaldehyde band remained the same. The R_f values of syringaldehyde in the control determination and in the lignin oxidation were identical (0.28). Thus, these findings once again give rise to the question of the possibility of a transition from a relatively low (15%) methoxylated lignin to a more highly (20%) methoxylated lignin in the process of lignification in some hardwoods.[14]

Klason[15] suggested that coniferyl alcohol could be the lignin progenitor in plant tissues. More recently, the formation of a lignin-like material by the action of mushroom phenol oxidase on coniferyl alcohol was reported.[16] It was thought[17] that this material is comparable with the native lignin from the softwood, black spruce. This has been taken as support for the contention concerning the biosyntheses of softwood lignin from coniferyl alcohol. If such were the case, it would necessarily follow that softwood lignins are made up completely of guaiacyl units.

However, in the light of the results presented herein, this cannot be stated unequivocally, otherwise it would follow that only guaiacyl type compounds would be obtained as softwood lignin degradation products. It has been found that the native lignin from the softwood, white Scots pine,

also yields *p*-hydroxybenzaldehyde upon oxidation. Thus, it seems that either coniferyl alcohol is not the only lignin progenitor in woody tissues, or that this coniferyl compound is preceded in the process of lignification by a simpler, less substituted aromatic system which in turn is formed from carbohydrates.

It has been demonstrated that the action of the "brown rot" mold, *Lentinus lepideus*, on glucose, xylose, or ethyl alcohol gives rise to methyl-*p*-methoxycinnamate.[18, 19] Acetaldehyde [20] was found to be the key intermediate in this enzymatic transition from carbohydrates to a methoxylated aromatic compound. Moreover, it is known that this mold is capable of overcoming the toxic effects of phenolic constituents through the process of methylation.[21] Thus it is a reasonable assumption that methyl-*p*-hydroxycinnamate is the primary metabolic product of the fungus. Hence, the mechanism of lignification can now be considered to be as follows: Carbohydrates are enzymatically transformed via a C_2 fragment to a *p*-hydroxyphenylpropane unit. This *primary* lignin progenitor then can undergo methoxylation to give a guaiacyl unit.[22] These two aromatic building stones in turn could form lignin by repeated condensations.

Evidence for this consideration lies in the fact that the basic structure of methyl-*p*-methoxycinnamate (I) is analogous to that of a *p*-hydroxy

I II

phenylpropane unit (II) from which *p*-hydroxybenzaldehyde could arise upon oxidation. This consideration supports and amplifies the experimental findings embodied in the phase sequence described previously.[11a, 23]

Enolizable Carbonyl Group in Lignin.—The results of the methylation of white Scots pine native and enzymatically liberated lignins with diazomethane and their phenylhydrazone derivatives are recorded in table 2. Since the methylated derivatives were still capable of forming phenylhydrazones, the groups methylated were not likely to be carbonyl functions in an enolic form. Further support for this was had by the absence of reaction between the isolated lignin and methanolic hydrogen chloride.[10]

The native lignin from white Scots pine had no absorption in the region above 300mμ, characteristic of the carbonyl group, but the hydrazone gave

a minor absorption peak at 352 mμ. Glading [24] accounted for the similar absorption spectra of spruce native lignin by assuming that any ketone groups were in an enolic form and absorption occurred at 352 mμ only when they were "fixed" in the hydrazone. However, it was found that the phenylhydrazone of diazomethane methylated lignin from white Scots pine gives some absorption in the region of 352 mμ. Thus, it appears that the

TABLE 2

DERIVATIVES OF WHITE SCOTS PINE NATIVE AND ENZYMATICALLY LIBERATED LIGNINS

	UNIT MOL. WT.	OCH_3, % CALC.	OCH_3, % OBS.	NO. OF GROUPS INTRODUCED
Native lignin (N. L.)	848[a]	..	14.5	..
Enzymatically liberated[b] Lignin (E. L.)	848	..	14.4	..
Lignin derivatives				
1. CH_2N_2 N. L.	862	18.0	18.5	1
2. CH_2N_2 E. L.	862	18.0	17.5	1
3. Phenylhydrazone of 1	952	16.4	16.0	1
4. Phenylhydrazone of 2	952	16.4	16.5	1

[a] Calculated on the basis of one carbonyl and four methoxyl groups per lignin building unit.

[b] Lignin liberated by *Lentinus lepideus*.

absorption in this region is not wholly dependent on whether or not the carbonyl group is enolized, but rather on the relative absorptive power of such a group. That is to say, the relative strength of the carbonyl group absorption in these native lignins is very weak (absence of conjugation) and thus is masked by the general absorption of the molecule. However, the phenylhydrazone derivative enhances the carbonyl absorption to the extent where it becomes detectable.

* Communication No. 269. The mold cultures used in this investigation were obtained through the courtesy of Dr. W. J. Robbins of the New York Botanical Garden. We wish to thank Professor Kei Arima of the University of Tokyo for his courtesy in supplying us with a sample of "kiri" wood. The analyses reported were carried out by Mr. A. A. Sirotenko, formerly of this Department. This work was carried out under the auspices of the Office of Naval Research.

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A PROPOSED STRUCTURE FOR THE NUCLEIC ACIDS

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The nucleic acids, as constituents of living organisms, are comparable in importance to the proteins. There is evidence that they are involved in the processes of cell division and growth, that they participate in the transmission of hereditary characters, and that they are important constituents of viruses. An understanding of the molecular structure of the nucleic acids should be of value in the effort to understand the fundamental phenomena of life.

We have now formulated a promising structure for the nucleic acids, by making use of the general principles of molecular structure and the available information about the nucleic acids themselves. The structure is not a vague one, but is precisely predicted; atomic coordinates for the principal atoms are given in table 1. This is the first precisely described structure for the nucleic acids that has been suggested by any investigator. The structure accounts for some of the features of the x-ray photographs; but detailed intensity calculations have not yet been made, and the structure cannot be considered to have been proved to be correct.

The Formulation of the Structure.—Only recently has reasonably complete information been gathered about the chemical nature of the nucleic acids. The nucleic acids are giant molecules, composed of complex units. Each unit consists of a phosphate ion, HPO_4^{--}, a sugar (ribose in the ribonucleic

acids, deoxyribose in the deoxyribonucleic acids), and a purine or pyrimidine side chain (adenine, guanine, thymine, cytosine, uracil, 5-methylcytosine). The purine or pyrimidine group is attached to carbon atom 1′ of the sugar, through the ring nitrogen atom 3 in the case of the pyrimidine nucleotides,[1] and the ring nitrogen atom 9 in the case of the purine nucleotides.[2] Good evidence has recently been obtained as to the nature of the linkage between the sugar and the phosphate, through the investigations of Todd and his collaborators;[3] it seems likely that the phosphate ester links involve carbon atoms 3′ and 5′ of the ribose or deoxyribose. New chemical evidence that the natural ribonucleosides have the β-D-ribofuranose configuration has also been reported by Todd and his collaborators,[4] and spectroscopic evidence indicating that the deoxyribonucleosides have the same configuration as the ribonucleosides has been obtained.[5] The β-D-ribofuranose configuration has been verified for cytidine by the determination of the structure of

TABLE 1

ATOMIC COORDINATES FOR NUCLEIC ACID

ATOM	ρ	ϕ	z	ATOM	ρ	ϕ	z
P	2.65 Å	0.0°	0.00 Å	O_1'	4.4 Å	45.4°	2.65 Å
O_I	2.00	28.3°	−0.67	O_2'	6.1	81.0°	2.1
O_{II}	2.00	−28.3°	0.67	N_3	6.7	52.8°	2.8
$O_{III} = O_5'$	3.72	13.5°	0.93	C_4	7.85	59.3°	2.8
$O_{IV} = O_3'$	3.72	−13.5°	−0.93	C_5	9.1	55.2°	2.8
C_5'	3.4	35.3°	0.7	C_6	9.35	46.9°	2.8
C_4'	3.2	51.6°	1.9	N_6	10.7	44.9°	2.8
C_3'	3.8	74.6°	1.55	N_1	8.45	39.9°	2.8
C_2'	5.3	70.3°	1.75	C_2	7.05	41.5°	2.8
C_1'	5.3	58.2°	2.8	O_2	6.35	32.4°	2.8

Identity distance along z axis = 27.2 Å.

Twenty-four atoms of each kind, with cylindrical coordinates (right-handed axes).

$\rho, \phi + n \cdot 105.0°, n \cdot 3.40 + z$; $\rho, \phi + n \cdot 105.0° + 120°, n \cdot 3.40 + z$; $\rho, \phi + n \cdot 105.0° + 240°, n \cdot 3.40 + z$; $n = 0, 1, 2, 3, 4, 5, 6, 7$.

the crystal by x-ray diffraction; cytidine is the only nucleoside for which a complete x-ray structure determination has been reported.[6]

X-ray photographs have been made of sodium thymonucleate and other preparations of the nucleic acids by Astbury and Bell.[7, 8] It has recently been reported by Wilkins, Gosling, and Seeds[9] that highly oriented fibers of sodium thymonucleate have been prepared, which give sharper x-ray photographs than those of Astbury and Bell. Our own preparations have given photographs somewhat inferior to those of Astbury and Bell. In the present work we have made use of data from our own photographs and from reproductions of the photographs of Astbury and Bell, especially those published by Astbury.[10] Astbury has pointed out that some information about the nature of the nucleic acid structure can be obtained from the x-ray photographs, but it has not been found possible to derive the structure from x-ray data alone.

A configuration of polypeptide chains in many proteins is the α helix.[11] In this structure the amino-acid residues are equivalent (except for differences in the side chains); there is only one type of relation between a residue and neighboring residues, one operation which converts a residue into a following residue. Through the continued application of this operation, a rotation-translation, the α helix is built up. It seems not unlikely that a single general operation is also involved in the construction of nucleic acids, polynucleotides, from their asymmetric fundamental units, the nucleotide residues. The general operation involved would be a rotation-reflection, and its application would lead to a helical structure. We assume, accordingly, that the structure to be formulated is a helix. The giant molecule would thus be cylindrical, with approximately circular cross section.

Some evidence in support of this assumption is provided by the electron micrographs of preparations of sodium thymonucleate described by Williams.[12] The preparation seen in the shadowed electron micrograph is clearly fibrous in nature. The small fibrils or molecules seem to be circular in cross-section, and their diameter is apparently constant; there is no evidence that the molecules are ribbon-like. The diameter as estimated from the length of the shadow is 15 or 20 Å. Similar electron micrographs, leading to the estimated molecular diameter 15 ± 5 Å, have been obtained by Kahler and Lloyd.[13] Also, estimates of the diameter of the molecules of native thymonucleic acid in the range 18 to 20 Å have been made[14, 15] on the basis of sedimentation velocity in the ultracentrifuge and other physicochemical data. The molecular weights reported are in the range 1 million to 4 million.

The x-ray photographs of sodium thymonucleate show a series of equatorial reflections compatible with a hexagonal lattice. The principal equatorial reflection, corresponding to the form 10·0, has spacing 16.2 Å or larger, the larger values corresponding to a higher degree of hydration of the substance. The minimum value,[7] 16.2 Å, corresponds to the molecular diameter 18.7 Å. From the average residue weight of sodium thymonucleate, about 330, and the density, about 1.62 g. cm.$^{-3}$, we calculate that the volume per residue is 338 Å.^{3} The cross-sectional area per residue is 303 Å^2; hence the length per residue along the fiber axis is about 1.12 Å.

The x-ray photographs show a very strong meridional reflection, with spacing about 3.40 Å. This reflection corresponds to a distance along the fiber axis equal to three times the distance per residue. Accordingly, the reflection is to be attributed to a unit consisting of three residues.

If the molecule of nucleic acid were a single helix, the reflection at 3.4 Å. would have to be attributed to a regularity in the purine-pyrimidine sequence, or to some other structural feature causing the three nucleotides in the structural unit to be different from one another. It seems unlikely

that there is a structural unit composed of three non-equivalent nucleotides.

The alternative explanation of the x-ray data is that the cylindrical molecule is formed of three chains, which are coiled about one another. The structure that we propose is a three-chain structure, each chain being a helix with fundamental translation equal to 3.4 Å, and the three chains being related to one another (except for differences in the nitrogen bases) by the operations of a threefold axis.

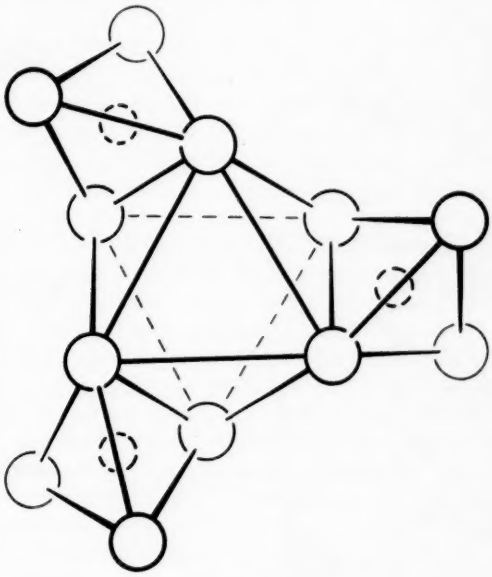
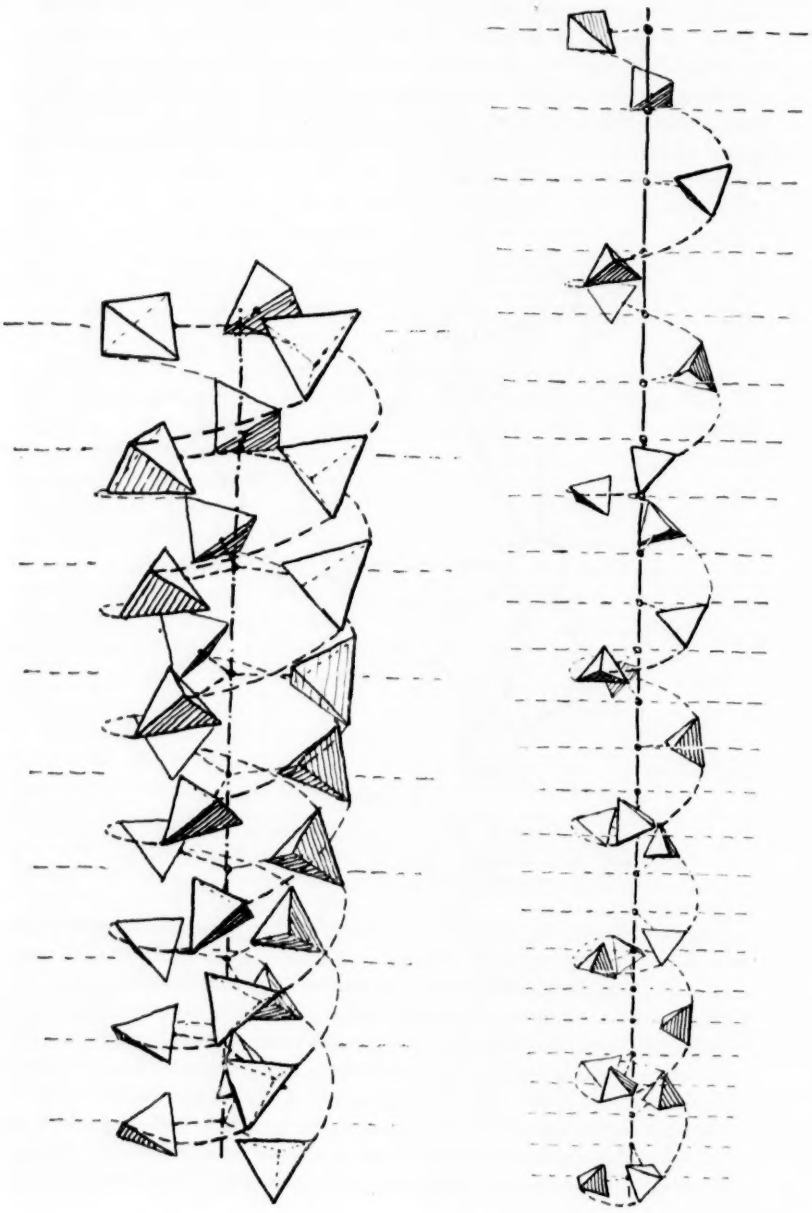


FIGURE 1

A group of three phosphate tetrahedra near the axis of the nucleic acid molecule. Oxygen atoms are indicated by full circles and phosphorus atoms by dashed circles.

The first question to be answered is that as to the nature of the core of the three-chain helical molecule—the part of the molecule closest to the axis. It is important for stability of the molecule that atoms be well packed together, and the problem of packing atoms together is a more difficult one to solve in the neighborhood of the axis than at a distance away from the axis, where there is a larger distance between an atom and the equivalent atom in the next unit. (An example of a helical structure which seems to satisfy all of the structural requirements except that of close packing of atoms in the region near the helical axis is the 5.2-residue helix of polypeptide chains. This structure seems not to be represented in proteins, whereas



the similar α helix, in which the atoms are packed in a satisfactorily close manner about the axis, is an important protein structure.) There are three possibilities as to the composition of the core: it may consist of the purine-pyrimidine groups, the sugar residues, or the phosphate groups. It is found by trial that, because of their varied nature, the purine-pyrimidine groups cannot be packed along the axis of the helix in such a way that suitable bonds can be formed between the sugar residues and the phosphate groups; this choice is accordingly eliminated. It is also unlikely that the sugar groups constitute the core of the molecule; the shape of the ribofuranose group and the deoxyribofuranose group is such that close packing of these groups along a helical axis is difficult, and no satisfactory way of packing them has been found. An example that shows the difficulty of achieving close packing is provided by the polysaccharide starch, which forms helixes with a hole along the axis, into which iodine molecules can fit. We conclude that the core of the molecule is probably formed of the phosphate groups.

A close-packed core of phosphoric acid residues, HPO_4^{--}, can easily be constructed. At each level along the fiber axis there are three phosphate groups. These are packed together in the way shown in figure 1. Six oxygen atoms, two from each tetrahedral phosphate group, form an octahedron, the trigonal axis of which is the axis of the three-chain helical molecule. A similar complex of three phosphate tetrahedra can be superimposed on this one, with translation by 3.4 Å along the fiber axis, and only a small change in azimuth. The neighborhood of the axis of the molecule is then filled with oxygen atoms, arranged in groups of three, which change their azimuthal orientation by about 60° from layer to layer, in such a way as to produce approximate closest packing of these atoms.

The height (between two opposite edges) of a phosphate tetrahedron is about 1.7 Å. If the same distance were preserved between the next oxygen layers, the basal-plane distance along the fiber axis would be 3.4 Å. This value is the spacing observed for the principal meridional reflection.

It is to be expected that the outer oxygen atoms of the complex of three phosphate groups would be attached to the ribofuranose or deoxyribofuranose residues, and that the hydrogen atom of the HPO_4^{--} residues

FIGURE 2

Figure 2 (*left*). A 24-residue 7-turn helix representing a single polynucleotide chain in the proposed structure for nucleic acid. The phosphate groups are represented by tetrahedra, and the ribofuranose groups by dashed arcs connecting them.

FIGURE 3

Figure 3 (*right*). One unit of the 3-chain nucleic acid structure. Eight nucleotide residues of each of the three chains are included within this unit. Each chain executes $3^1/_3$ turns in this unit.

would be attached to one of the two inner oxygen atoms, and presumably would be involved in hydrogen-bond formation with another of the inner oxygen atoms, of an adjoining phosphate group. The length of the O–H $\cdots$O bond should be close to that observed in potassium dihydrogen phosphate, 2.55 Å. The angle P—O—H should be approximately the tetrahedral angle. It is found that the spacing 3.4 Å is not compatible with this bond angle, if the hydrogen bonds are formed between one phosphate group

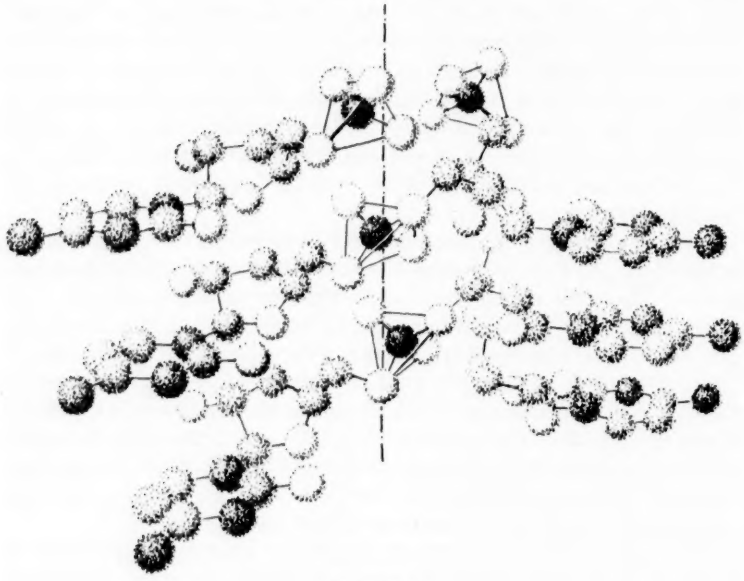


FIGURE 4

Perspective drawing of a portion of the nucleic acid structure, showing the phosphate tetrahedra near the axis of the molecule, the β-D-ribofuranose rings connecting the tetrahedra into chains, and the attached purine and pyrimidine rings (represented as purine rings in this drawing). The molecule is inverted with respect to the coordinates given in table 1.

and a group in the layer above or below it. Accordingly we assume that hydrogen bonds are formed between the oxygen atoms of the phosphate groups in the same basal plane, along outer edges of the octahedron in figure 1.

The maximum distance between the oxygen atoms 3′ and 5′ of a ribofuranose or deoxyribofuranose residue permitted by the accepted structural parameters (C—C = 1.54 Å, C—O = 2.43 Å, bond angles tetrahedral, with the minimum distortion required by the five-membered ring, one atom of

the five-membered ring 0.5 Å from the plane of the other four, as reported by Furberg[6] for cytidine) is 4.95 Å. It is found that it is very difficult to assign atomic positions in such a way that the residues can form a bridge between an outer oxygen atom of one phosphate group and an outer oxygen atom of a phosphate group in the layer above, without bringing some atoms into closer contact than is normal. The atomic parameters given in Table

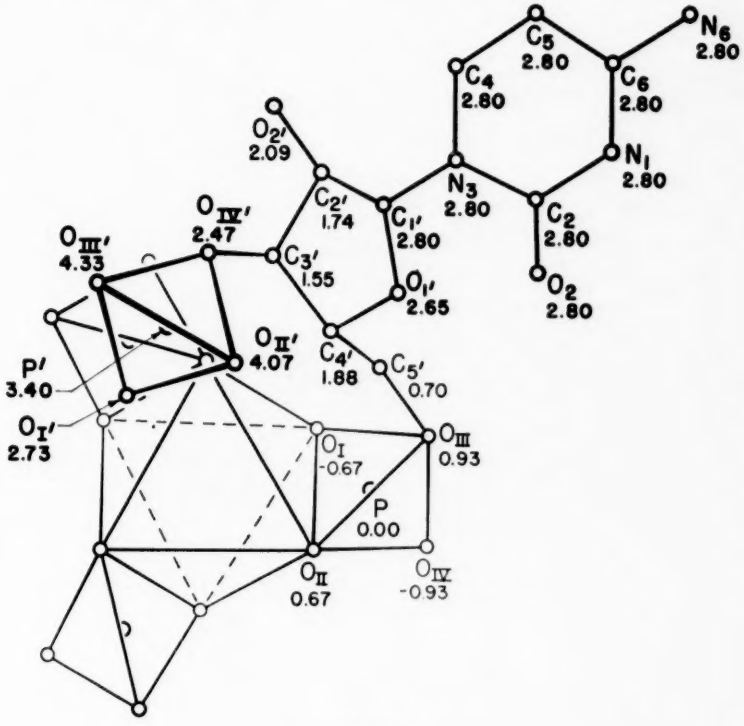


FIGURE 5

A plan of the nucleic acid structure, showing four of the phosphate groups, one ribofuranose group, and one pyrimidine group.

1 represent the best solution of this problem that we have found; these parameters, however, probably are capable of further refinement. The structure is an extraordinarily tight one, with little opportunity for change in position of the atoms.

The phosphate groups are unsymmetrical: the P—O distance is 1.45 Å for the two inner oxygen atoms, and 1.60 Å for the two outer oxygen atoms,

which are involved in ester linkages. This distortion of the phosphate group from the regular tetrahedral configuration is not supported by direct experimental evidence; unfortunately no precise structure determinations have been made of any phosphate di-esters. The distortion, which corresponds to a larger amount of double bond character for the inner oxygen atoms than for the oxygen atoms involved in the ester linkages, is a reason-

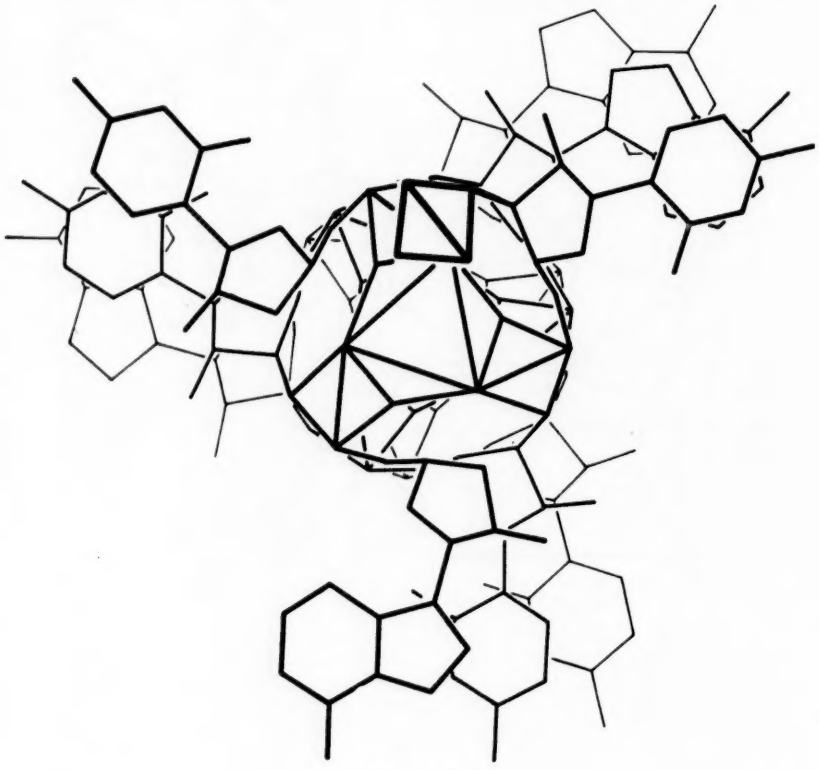


FIGURE 6

Plan of the nucleic acid structure, showing several nucleotide residues.

able one, and the assumed distances are those indicated by the observed values for somewhat similar substances, especially the ring compound S_3O_9, in which each sulfur atom is surrounded by a tetrahedron of four oxygen atoms, two of which are shared with adjacent tetrahedra, and two unshared. The O—O distances within the phosphate tetrahedron are 2.32 Å (between the two inner oxygen atoms), 2.46 Å, 2.55 Å, and 2.60 Å. The

hydrogen-bond distance is 2.50 Å, and each phosphate tetrahedron has two O—O contacts at 2.50 Å, with tetrahedra in the layer above. The group of three phosphate tetrahedra in each layer is obtained from that in the layer below by translation upward by 3.40 Å, and rotation in the direction corresponding to a left-handed screw by the azimuthal angle 15°. Thus there are strings of phosphate tetrahedra that are nearly superimposed, and execute a slow twist to the left. These strings are not connected together into a single polynucleotide chain, however. The sugar residues connect each phosphate group with the phosphate group in the layer above that is obtained from it by the translation by 3.40 Å and rotation through the azimuthal angle 105°, in the direction corresponding to a right-handed screw, as shown in figure 2. This gives rise to a helical chain, with pitch 11.65 Å, and with 3.43 residues per turn of the helix. The chain has an identity distance or approximate identity distance of 81.5 Å, corresponding to 24 nucleotide residues in seven turns, as shown in figure 3. The three chains of the molecule interpenetrate in such a way that the pitch of the triple helix is 3.88 Å, and the identity distance or approximate identity distance is 27.2 Å, corresponding to eight layers (see also Figs. 4, 5, and 6).

The structure requires that the sugar residues have the β-furanose configuration; steric hindrance would prevent the introduction of purine or pyrimidine groups in the positions corresponding to the α configuration. The planes of the purine and pyrimidine residues may be perpendicular or nearly perpendicular to the axis of the molecule. This causes these groups to be superimposed in layers that execute a slow left-handed turn about the molecule, the distance between the planes of successive groups being 3.4 Å. The orientation of the groups is accordingly that required by the observed strong negative birefringence of the nucleic acid fibers. The assignment of the sense of the helical molecules corresponding to the right-handed screw is required by the nature of the structure (the packing of the atoms near the axis, and the absolute configuration of the sugar, as given by the recent experimental determination[16] that absolute configurations are correctly given by the Fischer convention).

The structure bears some resemblance to the structures that have been suggested earlier, and described in a general way, without atomic coordinates. Astbury and Bell[7] suggested that the nucleic acid molecule consists of a column of nucleotide residues, with the purine and pyrimidine groups arranged directly above one another, in planes 3.4 Å apart. Astbury[10] considered the possibility that the nucleotides are arranged in a spiral around the long axis of the molecule, and rejected it, on the grounds that it does not lead to a sufficiently close packing of the groups, as is required by the high density of the substance. He pointed out that it is unlikely that adjacent molecules could interleave their purine and pyrimidine residues in such a way as to lead to the high density. Our structure solves this problem by

the device of intertwining three helical polynucleotide chains, in such a way that there are three nearly vertical purine-pyrimidine columns, consisting of purine and pyrimidine residues from the three chains in alternation. Furberg[17] suggested two single helical configurations, each resembling in a general way one of our helical polynucleotide chains, but his structures involve orientations of phosphate tetrahedra and the ribofuranose rings that are quite different from ours, and it is doubtful that three chains with either of the configurations indicated in his drawing could be intertwined.

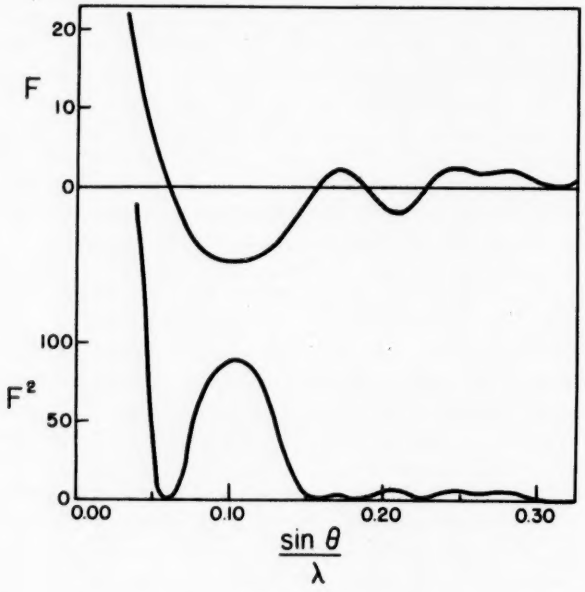


FIGURE 7

The calculated x-ray form factor F and its square F^2 for equatorial reflections of nucleic acid.

The proposed structure accounts moderately well for the principal features of the x-ray patterns of sodium thymonucleate and other nucleic acid derivatives. The spacing 3.40 Å between successive layers of three nucleotides along the molecular axis is required to within about 0.10 Å by the structural parameters of the nucleotides. The prediction that the helixes have 24 nucleotide residues per turn, corresponding to identity distance 8×3.4 Å in the direction of the fiber axis, is in good agreement with the fact that the x-ray diagrams can be reasonably well indexed by placing the 3.4 Å meridional reflection on the eighth layer line. The formula of Cochran,

Crick, and Vand[18] for the form factor for helical structures requires that the orders of Bessel functions for the successive layer lines from 0 to 8 be 0, 3, 6, 9, 12, 9, 6, 3, and 0. The layer-line intensities agree satisfactorily with this prediction, in the region from layer line 4 to layer line 8. There is an unexplained blackening near the meridian for layer lines 2 to 4, which, however, differs in nature for sodium thymonucleate and clupein thymonucleate, and which probably is to be attributed to material between the polynucleotide chains.

The distribution of intensity along the equator can be accounted for satisfactorily. In figure 7 there are shown the calculated form factor in the

TABLE 2

CALCULATED AND OBSERVED EQUATORIAL X-RAY REFLECTIONS FOR SODIUM THYMONUCLEATE. HEXAGONAL UNIT WITH a_0 = 22.1 Å

hkl	$d_{calc.}$	F_1	F_2	pF_2^2	$I_{obs.}$[a]	$d_{obs.}$[a]
10.0	19.1 Å	55	47	6600	m	18.1 Å
11.0	11.0	9.6	21	1350	m	11.2
20.0	9.5	4.7	−1.0	3		
21.0	7.22	−3.4	−8.9	480	w	7.16
30.0	6.37	−7.7	3.1	29		
22.0	5.52	−9.2	1.3	5		
31.0	5.30	−9.4	−14.6	1280	m	5.30
40.0	4.78	−9.3	−14.4	620		
32.0	4.38	−9.1	−14.1	1200	m	19[b]
41.0	4.17	−8.8	1.0	6		
50.0	3.83	−6.1	−10.8	350		
33.0	3.68	−5.1	4.2	53		
42.0	3.61	−4.3	−8.9	480	vw	3.57
51.0	3.43	−2.6	−7.1	300		

The symbol p in column 5 is the frequency factor for the form.

[a] The observed intensity values and interplanar distances are those reported by Astbury and Bell.

[b] The reflection covers the angular range corresponding to interplanar distances 4.0 to 4.4 Å, and may arise in part from overlapping from the adjacent layer lines.

equatorial direction, and the square of the form factor. It is seen that the form factor vanishes at a spacing of about 8 Å, and has a maximum in the region near 5 Å. Calculated intensities, given in table 2, are obtained by making a correction for interstitial material, at the coordinates 1/3 2/3 and 2/3 1/3, the amount of this material being taken as corresponding in scattering power to 1.5 oxygen atoms per nucleotide residue. There is reasonably satisfactory agreement with the experimental values; on the other hand, similar agreement might be given by any cylindrical molecule with approximately the same diameter. A comparison of observed and calculated radial distribution functions would provide a more reliable test of the structure; this comparison has not yet been carried out.

It is interesting to note that the purine and pyrimidine groups, on the periphery of the molecule, occupy positions such that their hydrogen-bond forming groups are directed radially. This would permit the nucleic acid molecule to interact vigorously with other molecules. Moreover, there is enough room in the region of each nitrogen base to permit the arbitrary choice of any one of the alternative groups; steric hindrance would not interfere with the arbitrary ordering of the residues. The proposed structure accordingly permits the maximum number of nucleic acids to be constructed, providing the possibility of high specificity. As Astbury has pointed out, the 3.4-Å x-ray reflection, indicating a similar distance along the axis of the molecule, is approximately the length per residue in a nearly extended polypeptide chain, and accordingly the nucleic acids are, with respect to this dimension, well suited to the ordering of amino-acid residues in a protein. The positions of the amino-acid residues might well be at the centers of the parallelograms of which the corners are occupied by four nitrogen bases. The 256 different kinds of parallelograms (neglecting the possibility of two different orientations of each nitrogen base) would permit considerable power of selection for each position.

(Added in proof.) Support of the assumed phosphorus-oxygen distances in the phosphate di-ester group is provided by the results of the determination of the structure of ammonium tetrametaphosphate.[19, 20] In this crystal there are P_4O_{12} complexes, consisting of four tetrahedra each of which shares two oxygen atoms with other tetrahedra. The phosphorus-oxygen distance is 1.46 Å for the oxygen atoms that are not shared, and 1.62 Å for those that are shared. These values are to be compared with the values that we have assumed, 1.45 Å for the inner oxygen atoms (which are not shared), and 1.60 Å for the outer ones, which have bonds to carbon atoms.

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*INDUCED DOMINANT LETHALITY IN LILIUM**

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In 1927, H. J. Muller[1] noticed a marked decrease in the fertility of female *Drosophila melanogaster* mated to irradiated males, and attributed this decrease to dominant lethals induced by the x-ray treatment.

As pointed out by Stancati,[2] in 1932, the primary choice under such circumstances is between the induction of dominant lethals and the inactivation of sperm. Either effect of x-rays would give the observed decrease in fertility. Although Muller and Settles[3] had proved in 1927 that sperm could function normally even though carrying chromosome sets which would be lethal in combination with the normal chromosome complement, Stancati's work with *Habrobracon* was the first to demonstrate conclusively that dominant lethality was the result, not the lack, of fertilization by irradiated sperm. After treatment, the frequency of the biparental females (arising from fertilized eggs) decreased while the frequency of the matroclinous, uniparental males (arising from unfertilized eggs) was neither diminished nor increased. The missing biparental females were those which would have appeared if dominant lethals had not been induced in the sperm.

Later Demerec and Kaufmann[4] examined cytologically Drosophila eggs presumably fertilized by sperm treated with 5000 r. Failure of fertilization was apparent in only one case out of the 99 studied. Therefore the high degree of sterility at this dose was assignable to dominant lethality rather than sperm inactivation. On the other hand, any studies of dominant lethality at yet higher doses would again require that the sperm be shown to act in fertilizing.

Work with *Habrobracon* also gave information on the dosage necessary to achieve sperm inactivation. At the relatively low doses generally used, the

frequency of biparental females decreases with increasing dosage while the frequency of uniparental males remains constant and equal to that of the controls. If sperm were inactivated, however, the frequency of unfertilized eggs would obviously increase, resulting in an increase of matroclinous males. Using this reasoning, Jane Maxwell[5] concluded that the increase in matroclinous males at the relatively high dose of 142,000 r was due, therefore, to partial inactivation of *Habrobracon* sperm at this dose.

In plants, more than very slight departures from the normal chromosome complement, particularly deficiencies, will markedly affect the formation and function of the pollen grain. In addition, much literature exists on the competitive relationships between types of pollen differing only slightly in genetic constitution. Such cases range from those of strictly determined self-sterilities to very slight departures from Mendelian expectancy in certain examples of selective fertilization.

In view of these considerations, failure of seed set following treatment of pollen with mutagenic agents cannot be assumed, *a priori*, to be due to the induction of dominant lethals. In *Zea Mays*, induction of a dominant lethal can be assumed when either the endosperm or the embryo alone is seriously deranged or absent; the existence of some seed tissue is assurance that fertilization has occurred.[6, 7] The idea that pollen inactivation soon leads to much of the observed sterility is implied by the remarks of Stadler and Uber[8] on the results of ultra-violet treatments, "The number of pollen grains applied is considerably in excess of the number of silks pollinated, but with increasing dosage the number of pollen grains capable of accomplishing fertilization declines until with the largest practical doses only a few seeds are produced on each ear."

The results to be previewed here demonstrate that such assumptions, although perhaps true for the ultra-violet treatments, cannot be made for a specific mutagen without further test. Pollinations with lily pollen treated with 2000 to 4000 r-units of x-rays result in little, or usually no seed set. It may easily be demonstrated in lily, however, that the failure of the ovules to develop is due to the induction of dominant lethals rather than failure of the irradiated grains to achieve fertilization.

The method to be described was developed as a means of exploring two problems of interest in this laboratory: (1) the chemical induction of mutations, and (2) comparison of the damage done by x-rays to the functional activity of the pollen with that done to the heritable component.

P. W. Whiting[9] has called attention to the utilization of dominant lethality in *Habrobracon* as an index of the mutagenic effectiveness of physical agents. In lily, dominant lethality can be studied also with chemical agents: normal seed sets have been obtained after pollinations with pollen handled in sucrose solutions, or phosphate buffers at lower pH's. Some fertilizations have followed treatments with phosphate buffers at higher

pH's, and solutions of colchicine or detergent. No information is yet available as to the mutagenic action on lily pollen of any of these agents.

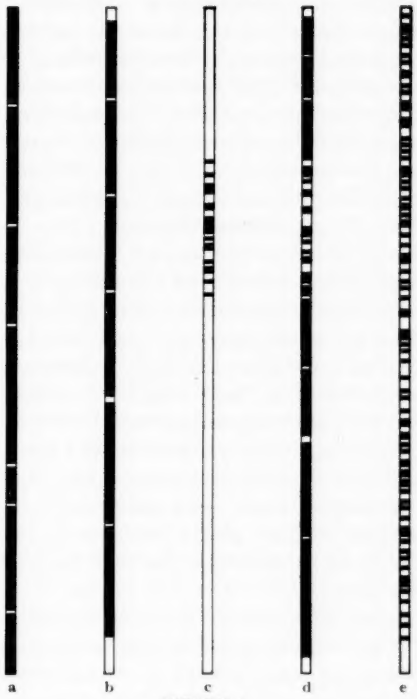

FIGURE 1

Diagrams of seed sets in single rows of seeds after various pollinations. The black bars and regions indicate seeds, the white bars and regions indicate empty ovules (or occasional germless seeds in the treated series). *a* and *b*, massive, untreated; *c*, limited pollination, untreated pollen; *d*, limited pollination, 4000 r-pollen, repeat after 48 hrs., massive, untreated; *e*, 4000 r-pollen, untreated pollen, each massively on two halves of stigma. (These diagrams are generalizations, not averages, based on several examples of each type.)

In regard to the second problem, one method of determining the functional activity of the pollen is identical with that for the detection of dominant lethals. In addition, studies of direct competition between treated and untreated grains allow for detection of slight impairment of function.

Results of Experiments.—The capsule of *Lilium formosanum* contains a total of about 1200 ovules arranged in two rows in each of three locules, or about 200 ovules per row. After a massive pollination of untreated pollen, seed is usually set along the entire length of each row (Fig. 1 (*a*)). In some instances, the ovules at the bottom or top of each row may not develop (Fig. 1 (*b*)). If pollinated, however, with a limited amount of pollen, the seed is not scattered along the row at random but is concentrated in a "favored zone" which centers about one-third of the way, as determined by serial counts of ovules, from the top to bottom of the capsule (Fig. 1 (*c*)). Such favored zones in plant ovaries have been recognized for years, and have been particularly studied in various Onagraceous genera.[10]

If pollinated massively following a treatment of 4000 r, the standard dose for the work summarized here, the capsule is typically devoid of mature seeds. The question here is that stated in the introduction: were dominant lethal effects induced, or was fertilization prevented by damage to the grain induced by the x-rays?

This question was answered by pollinating first with a limited, counted number of treated pollen grains and repollinating massively with normal grains after an interval of 48 hours (the x-ray-repeat-normal pollinations). Capsules from such repeat pollinations had aborted ovules in the favored zone and these were surrounded, top and bottom, by normal seeds (Fig. 1 (*d*)). This experiment thus demonstrated that fertilizations occurred and that dominant lethals had, therefore, been induced. On comparing the number of pollen grains applied with the number of aborted ovules later appearing in the favored zone, it was possible to determine that the treated grains functioned to give fertilizations at almost the same percentages, 90–95%, as grains handled similarly but not irradiated.

A control of the repeat pollination experiment was made by pollinating first with a limited number of normal grains followed 48 hours later by a massive pollination of normal pollen. The full sets of seed obtained in this experiment indicated that the stigma was not rendered unreceptive by the primary pollination, nor was seed set otherwise detectably influenced. In a further control, flowers of greater age than those used in the repeat pollination experiment proved to have receptive stigmas and healthy ovules. Their petals had fallen and many of the stigmas had turned brown; nonetheless, full sets of seed were obtained in the upper one-half to two-thirds of the capsules, always including the favored zone. This experiment indicated that the presence of undeveloped ovules in the favored zone in the x-ray-repeat-normal pollinations was not attributable to their spoilage from aging during the 48-hour time lapse.

Sections of ovaries fixed several days after massive pollinations with x-ray treated pollen indicated presence of pollen tubes in all micropyles and two sperm nuclei in the sac. Fixations after longer intervals from pollination have shown the types of necrotic effects present, usually following several cell divisions.

The presence of some germless seeds after various doses of x-rays is evidence that the endosperm of lily is capable of developing alone in some instances. The fact that it can sometimes be seen to undergo necrotic changes before the zygote begins to divide is evidence that the genetic changes present in the haploid sperm can be immediately dominant over the four normal complements provided maternally. The question as to possible later interactions between damaged embryo and damaged endosperm cannot now be answered.

By applying untreated pollen on half the stigma and immediately thereafter applying treated pollen to the other half, the treated grains can be put in direct competition, as to functional capacity, with the normal grains. Following such half-and-half pollinations, the normal seeds and aborted ovules alternate at random within the rows (Fig. 1 (*e*)). There is a decided tendency for one locule to have a preponderance of normal seeds, another

to have a preponderance of aborted ovules, while the third has a more nearly equal mixture of the two types. The application of small amounts of pollen, either normal or treated, to restricted sections of the stigma will also give rise to unequal distributions among the three locules, but never as markedly unequal as those noted in these half-and-half pollinations. The results of the half-and-half experiment indicate that the treated grain is not functionally handicapped in competition with the normal. There is certainly no tendency for the normal seeds to be concentrated in the favored zone.

In one experiment, several massive pollinations after 4000 r-treatments gave some seed set; in one case, the unexpectedly high frequency of 13% was noted. Because of the very low seed set following doses of 1500 and 2000 r, the latter sometimes giving no normal seeds, and the usual complete lack of seeds after the standard treatment of 4000 r, either contamination or pseudogamy seemed required for an explanation. The flowers in the greenhouse were all emasculated routinely; contamination seemed unlikely. Pseudogamy has been reported to occur in *Lilium* following species crosses;[11] the number of seeds developed was, however, variable, and seemed dependent upon environment. Pseudogamy therefore appeared to be the more likely explanation for the exceptional seed sets. It is unlikely, however, that pseudogamy is a factor affecting appreciably the results obtained following the x-ray-repeat-normal pollinations. The frequency of seeds in those cases which might be attributed to pseudogamy have been too low, on the average, to be of significance in the repeat pollinations.

The problem of pseudogamous development was explored further in semi-male-sterile types appearing in one of the inbred lines being maintained in this laboratory. The semi-male-sterile condition is different from the semi-sterilities caused by chromosomal aberrations such as translocations and inversions; usually the anthers produce no good pollen, but sometimes one region of an anther will yield some good pollen, and some seeds can be obtained on self-pollinating. The unusual vigor apparent in this culture in seedling stages in the F_2 generation of inbreeding was considered to be a possible indication of pseudogamous development.

Plants of the semi-male-sterile type will set no seed unless pollinated, and will set full capsules if pollinated routinely with normal, untreated pollen. After pollinations with irradiated pollen, the capsules will regularly contain small percentages of normal seeds, these percentages being about the same as those appearing in the few exceptional cases following pollinations of non-male-sterile plants with treated pollen. It would thus seem likely that the occasional low seed set after the 4000 r-pollinations of the non-male-steriles is due to occasional pseudogamy rather than to contamination or to lack of complete dominant lethality in the embryo.

On the other hand, the low frequency of good seed after treated pollinations of the semi-male-sterile types may be indicative of an embryo-endosperm interaction; thus a relatively undamaged endosperm may be necessary for the development of an adventitious embryo. Embryological examinations following x-ray pollinations of the semi-male-sterile types is also expected to give further information on pseudogamy.

Summary.—After relatively low doses of x-rays (2000 to 4000 r) applied to the pollen of *Lilium formosanum*, few or no seeds are set. Failure of the ovules to develop is due to the presence of dominant lethals in the gametes contributed by the treated pollen. In competition with untreated pollen, the treated grains are not handicapped in their ability to produce pollen tubes and achieve fertilizations.

Occasionally a few seeds are set at doses which otherwise consistently give complete dominant lethality. These seeds are probably the product of pseudogamous development. The problem of pseudogamy is being explored further in semi-male-sterile types which usually yield a few seeds at doses expected to be completely lethal.

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KAPPA CONCENTRATION PER CELL IN RELATION TO THE LIFE CYCLE, GENOTYPE AND MATING TYPE IN PARAMECIUM AURELIA, VARIETY 4*

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Kappa, the physical basis of the killer trait in Paramecium, can be maintained by paramecia of variety 4 (under standard cultural conditions) only when the nucleus contains gene *K;* in homozygotes for the allele *k*, it disappears.[1] As kappa is a microscopically visible particle of 0.2 to 0.8 μ diameter,[2] it may be directly observed as an index of activity of the genes *K* and *k*. Specifically, killers containing many kappa particles may, by crosses, have their *K* genes replaced by *k*, and quantitative studies may then be made of the manner in which kappa subsequently disappears. This and related studies should yield evidence on the way in which the effects of a prior genotype wear off (a phenomenon known variously as maternal effect, phenomic lag, and cytoplasmic lag) and the way in which a newge notype comes into action.

In the course of studies of this sort, it became apparent that cellular concentration of kappa varies according to a definite pattern during the life cycle and that the concentration level at comparable stages of the cycle were characteristically different in different genotypes and mating types. These discoveries not only extended the possibilities of utilizing kappa as a measure of gene action, but provided a clue to the mechanism of mating type determination (see Nanney[13]).

Methods.—Experimental animals were grown in depression slide cultures at 27°C. Daily isolations were made in order to maintain the animals at maximum fission rate, i.e., about 5 fissions per day. See Sonneborn[3] for methods of culture, crossing, inducing autogamy, and so on.

For the study of kappa, animals free of food vacuoles are most suitable, for bacteria in food vacuoles may confuse the kappa counts. Keeping the paramecia in sterile culture fluid for $1^1/_2$ hours before fixation effectively frees them of food vacuoles. In order to obtain paramecia close to a standard stage of the fission cycle, the paramecia to be stained were isolated into sterile fluid 3 hours after each of the first 3 post-fertilization fissions and at 5-hour intervals thereafter, since the interfission period is about 5 hours.

After the period in sterile fluid, animals were fixed with Schaudinn's fixative and stained according to Dippell and Chao's modification of DeLamater's basic fuchsin technique.[3] Observations were carried out at a magnification of 900 ×. Inserted in the ocular of the microscope was a glass grid with its squares of dimensions such that the image of an animal

occupied approximately 15 of them. The kappa particles appearing in each square were counted and recorded, the sum giving the total for the whole animal. Absolute accuracy of counts cannot be obtained. The degree of accuracy was estimated by making recounts on some of the animals after an interval of 6 months. The animals for recounts were selected by other investigators and the original records at that time were not in the possession of the author. Recounts never differed from the original counts by more than 10%.

Materials.—Two cultures of variety 4 were employed in this investigation, the killer stock 51 and an experimentally derived sensitive culture d186. The latter is to a large extent isogenic with stock 51 but bears the gene *k* from stock 29 instead of gene *K* of stock 51.[4]

The Relation of Kappa Concentration to Stages of the Life Cycle.—The variations of kappa concentration in the animal during its life cycle were investigated in animals of genotypes *KK* and *Kk* of both mating types, VII and VIII. The homozygous *KK* animals of both mating types were obtained by inducing autogamy in the left-over animals from daily isolation lines. Autogamous animals were singly isolated into depression slides. After the 1st to the 14th, except the 5th, fission for the homozygous *KK* VIII and 1st to 12th fission for homozygous *KK* VII, one of the products of each division in each of the cultures was fixed, stained, and observed for its quantity of kappa. The data for the type VIII homozygous (*KK*) killers were obtained from two experiments. The first experiment provided data for the 1st to the 6th fission and the second experiment provided data for the 7th to the 14th fission.

Heterozygous *Kk* animals of both mating types were obtained by cross ing homozygous *KK* killers of the 2 mating types from stock 51 to sensitives (*kk*) of the complementary mating type from culture d186. Both conjugants of each pair acquire the genotype *Kk;* but in the absence of cytoplasmic exchange between the mates, only the killer member of a pair produces a clone of killers. The mating types of the 2 exconjugants usually remained the same as they were before conjugation. Hence, in order to obtain heterozygous killers of type VII, the killer member in the cross was mating type VII; and the reciprocal cross was employed to obtain *Kk* killers of the type VIII. After the heterozygous F1 animals obtained from the two members of each pair had undergone about 15 fissions after conjugation, they were crossed to each other. This cross produced a 1:2:1 ratio of homozygous *KK* killers, heterozygous *Kk* killers and homozygous *kk* sensitives from the killer member of the F1 pair. These 3 kinds of animals cannot be identified by their kappa contents before the 7th fission; therefore a mixture of clones of these 3 kinds of animals was used for the study of kappa during the first 6 fissions of the life cycle and pure *Kk* killers derived from a single clone were used for later fissions (7th to 14th). Heterozy-

gosity of the F2 killers was verified by obtaining from them an F3 generation by autogamy and verifying the expected segregation of killers (KK) and sensitives (kk) in 1:1 ratio.

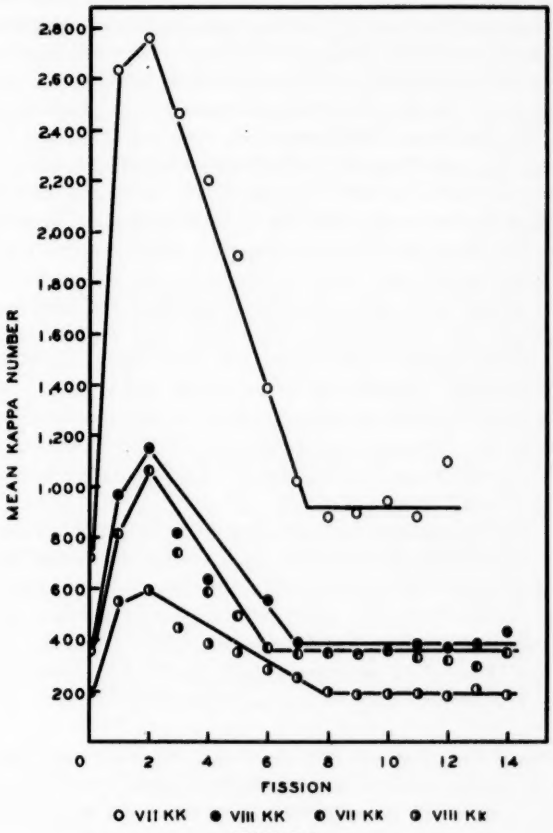

FIGURE 1

Variation of kappa number with the stage of the life cycle. The mean kappa number per cell is plotted against the number of fissions since autogamy and conjugation. The kappa means are for 10 exautogamous or exconjugant cultures of KK and Kk of mating type VII and VIII.

The means of counts of kappa in animals of the 2 genotypes of each mating type are plotted in figure 1. All points on these curves, except those for zero fissions, were obtained in the way set forth in the preceding paragraphs. The kappa counts at zero fissions for the heterozygous (Kk) type

VII animals were made at the crescent stage of the 1st meiotic division of conjugation, in a cross between F1 *Kk* type VII killers and F1 *Kk* type VIII killers. The same clone of type VII *Kk* killers was used for the life cycle study. The kappa counts for zero fissions for the other 3 kinds of animals were also obtained from animals at the same stage of conjugation. The crosses employed were homozygous (*KK*) type VII to homozygous (*KK*) type VIII for homozygous (*KK*) killers of both mating types, VII and VIII, and heterozygous (*Kk*) type VII to heterozygous (*Kk*) type VIII for heterozygous VIII. But these killers were not from the same experiments as were used for the later stages in the life cycle of these 3 kinds of animals.

Figure 1 shows for all 4 kinds of cultures that there are 3 stages during the life cycle differing in concentration of kappa per cell. During the 1st stage, extending from soon after fertilization until the 2nd fission after fertilization, the amount of kappa increases. It rises almost to the peak after the 1st fission but increases a little more after the 2nd fission. At the peak, the animals contain approximately 3 times the amount of kappa present before fertilization. The 2nd stage is marked by the reduction in the amount of kappa and extends from the 3rd fission to the 7th fission. By the end of this period, the amount of kappa has returned to the pre-fertilization level. It remains there throughout the 3rd stage, extending from the 8th fission to the 14th fission (and, presumably, later fissions), when the next fertilization can occur.

The pattern of the changes in kappa concentration per cell is the same for the animals with different combinations of genotype and mating type. The means at the peak of kappa concentration per cell, attained at the end of the 1st stage of the life cycle, i.e., after the 2nd post-fertilization fission, are: for homozygous (*KK*) type VII killers about 2700 particles; for heterozygous (*Kk*) VII and the homozygous (*KK*) type VIII animals about 1100 particles, and for heterozygous (*Kk*) type VIII killers about 600 particles of kappa.

During the 3rd stage of the life cycle, when each kind of killer animal maintains its kappa concentration, the heterozygous type VII animals and homozygous type VIII animals both have means of approximately 400 particles, but the homozygous type VII cells have roughly twice as much and the heterozygous type VIII cells roughly half as much. In other words within each mating type, the homozygotes have about twice as much kappa as the heterozygotes; and genotype for genotype, the type VII cells have about twice as much kappa as the type VIII cells.

The question of whether these observations are repeatable is answered by the data of table 1, which summarizes numerous repetitions of the counts on the animals in various parts of the 3rd stage of the life cycle, from the beginning (the 8th fission) until the next fertilization. On the basis of the data it may be concluded that the mean number of kappa particles per cell under

the conditions of these experiments is regularly about 200 for *Kk* type VIII, 400 for *KK* type VIII and *Kk* type VII, and 800 for *KK* type VII. Counts made under different cultural conditions or at different stages of the fission cycle give different values. This will be set forth in a later paper.

These numerical relations were not anticipated, although the finding of the higher counts for mating type VII animals might have been expected. Preer[5] and Sonneborn[6, 7] had previously remarked that intensity of killing activity decreases with experimentally induced decrease in kappa concentration. Further, Sonneborn[8] and Austin[9] had noticed that type VII animals are stronger killers than type VIII animals. Hence one might have deduced that there is more kappa in type VII animals than in type VIII animals, but this conclusion had never been drawn.

TABLE 1

STANDARD ERROR OF MEANS FOR THE KAPPA COUNTS AT THE 3RD STAGE IN THE LIFE CYCLE FOR *KK* AND *Kk* KILLERS OF TYPES VII AND VIII

(*a*) Means and standard error, s.e., for the means for various experiments

MATING TYPE	GENOTYPE	MEANS FOR VARIOUS EXPERIMENTS	MEAN ± S.E.
VII	*KK*	674, 679, 775, 822, 855, 861, 897, 915	810 ± 34
	Kk	332, 406, 414, 420, 437	402 ± 18.3
VIII	*KK*	327, 337, 339, 361, 369, 371, 377, 379, 386, 388, 393	366 ± 6.8
	Kk	184, 193, 197, 197, 199, 200, 202, 210	198 ± 2.65

(*b*) Means and standard error for all the animals included in the experiments in (*a*)

MATING TYPE	GENOTYPE	NUMBER OF ANIMALS	NUMBER OF KAPPA PARTICLES	
			MEAN ± S.E.	RANGE OF VARIATION
VII	*KK*	102	851 ± 15	497–1303
	Kk	107	363 ± 6.8	219–611
VIII	*KK*	147	371 ± 4.6	240–634
	Kk	134	195 ± 2.3	140–270

Changes of Kappa Concentration Following Change of Mating Type.— Since mating type seldom changes in variety 4 after fertilization, the question arises as to whether the different kappa levels found in the 2 mating types are actually due to difference of mating type or whether they are merely clonal differences and are independent of mating type. This question was answered by taking advantage of the fact that mating type does occasionally change at fertilization. A clone pure for type VIII was induced to undergo autogamy and the resulting culture was observed for selfing, which would of course follow the origin of cells of type VII. A pair of conjugants was in time found and removed from the culture. The 2 exconjugants were separately cultivated and, as expected, one proved to be type VIII, the other type VII. Cells of these two cultures were than stained for kappa counts. The means for these counts were 327 for the type

VIII culture and 678 for the type VII culture. Although both clones gave counts somewhat lower than the means for the homozygotes of all experiments taken together (table 1), again the type VII mean is about twice as great as the mean for type VIII. Since both of these cultures were derived a short time earlier from a single ancestral cell, the difference between them is clearly a consequence of the change of mating type. Details of the rate and manner of change of kappa concentration following change of mating type will be reported in a later paper.

Change of Kappa Concentration Following Change of Genotype.—Because the amount of kappa is smaller and more readily counted in type VIII animals, they alone were used to study in detail the time and manner of change of kappa concentration following change of genotype. Two changes were examined: from *Kk* killers to *KK* killers and to *kk* sensitives. Both were followed in parallel by starting with *Kk* killers and allowing them to undergo autogamy. There result 2 kinds of clones: *KK* killers and *kk* sensitives in a 1:1 ratio. The changes in kappa concentration were followed through the first 15 fissions after autogamy. In one experiment, the 2 kinds of clones were not separated, since they cannot be identified at first. It was expected that the kappa counts would quickly show a bimodal distribution; but this did not begin to appear before the 9th fission. The study was then repeated using isolated single clones, each stained separately and later identified as to genotype and phenotype. In this experiment, the 2 kinds of cultures (*KK* and *kk*) were stained only from the 7th to the 15th fissions. Table 2 gives the results of the first experiment for the first 6 fissions; the results of the second experiment for the later fissions.

As appears in table 2, these 2 groups of animals (*KK* and *kk*) cannot be distinguished from one another by their kappa counts before the 8th fission. The counts do not show a bimodal distribution as late as the 6th fission, when they were separated and individually identified. On the contrary, up to this point the 2 kinds of animals are alike in their kappa concentration which follows the course typical for the first 2 periods of the life cycle shown in figure 1, but at the level characteristic of heterozygotes of type VIII.

Eventually, however, this level shifted to those characteristic of the 2 kinds of homozygotes. In the *KK* homozygotes, the change took place suddenly and dramatically within one fission cycle in every cell examined. After the 8th fission, no *KK* cell had more than 284 kappa particles; after the 9th fission none had less than 308. In the course of one fission cycle the mean rose from 223, a characteristic heterozygous level, to 339, a characteristic homozygous level. Detailed examination of the individual counts shows that at the 7th and 8th fissions a few of the animals (even in the *kk* group), possibly all those with more than 250 kappa particles and almost certainly those with more than 270 particles, had not yet dropped down to the heterozygous level. This tends to exalt the means and reduce the ap-

TABLE 2

KAPPA COUNTS FOR THE F_2 OBTAINED BY AUTOGAMY FROM *Kk* KILLERS OF MATING TYPE VIII

FISSION GENOTYPES	1 *KK* *kk*	2 *KK* *kk*	3 *KK* *kk*	4 *KK* *kk*	5 *KK* *kk*	6 *KK* *kk*	7 *KK*	7 *kk*	8 *KK*	8 *kk*	9 *KK*	9 *kk*	10 *KK*	10 *kk*	11 *KK*	11 *kk*	12 *KK*	12 *kk*	13 *kk*	14 *kk*	15 *kk*
Kappa counts	397	429	276	306	241	162	141	157	177	0	308	0	267	0	247	0	266	0	0	0	0
	408	444	308	328	272	189	164	171	199	125	310	4	340	27	299	0	272	0	0	0	0
	427	490	382	339	307	209	183	178	212	180	311	109	343	28	339	16	306	0	0	0	0
	427	522	413	341	328	218	185	179	214	185	314	112	362	86	339	46	316	0	0	0	0
	429	526	421	346	331	222	214	184	223	188	333	152	372	138	357	58	345	0	0	0	0
	449	543	448	349	332	234	219	195	242	201	336	152	409	157	361	59	348	4	5	4	0
	451	563	461	367	334	266	243	224	247	230	342	200	489	169	388	73	369	49	32	15	0
	466	568	487	393	357	293	244	266	263	235	355	222	489	194	452	117	379	94	63	17	0
	609	579	517	433	362	338	325	269	273	256	378	224	495	196	458	156	396	108	78	27	0
	619	600	531	464	430	355	339	305	280	284	409	241	519	216	496	164	406	146	98	79	0
Mean	478	523	424	366	323	249	225	212	223	188	339	141	398	121	374	69	341	40	27	14	0

parent magnitude of the sudden rise in the *KK* animals at the 9th fission; but there is no question that all of the animals rose to within the range characteristic of the *KK* level and maintained it thereafter. Perhaps examination of a larger sample would have revealed some which failed to change over completely at the 9th fission; nevertheless the regularity of the occurence of the change at this stage under the conditions of this experiment can hardly be doubted. It is as if the genotype, previously inoperative on kappa concentration, suddenly comes into full and complete expression.

The changes in the *kk* animals follow a very different course. At each fission, beginning with the 8th, some of the cells lose all of their kappa, others lose half or more of their kappa, and others retain the heterozygous level of 150 or more particles. In the groups of 10 animals counted after each fission, 2 showed a drop in kappa after the 8th fission, 2 more (total 4) after the 9th, one more after the 10th, 3 more after the 11th. Only one was still at the heterozygous level after the 12th fission, none after the 13th; but some kappa was still retained by a number of the cells even after the 14th fission. Thus, loss of kappa in the *kk* animals may be as sudden and complete as its rise in the *KK* cells; but it does not occur uniformly in all cells at the same fission. On the contrary, the loss of kappa is spread out over a period of 8 fissions.

From the data of table 2, it can be calculated that the kappa in a *kk* exautogamous animal increases in the progeny of this cell on the average to an amount 2^{10} to 2^{11} times that initially present before kappa entirely disappears. Perhaps the long persistence and multiplication of kappa in the *kk* animals is due in part to activity of the fragments of the previous *Kk* macronucleus, one or more of which may be present in the cells as late as the 5th or 6th fission. Whatever the explanation, it seems clear that the loss of activity of the previous *Kk* nucleus in the *kk* cells follows a very different course from that manifested by coming into action of a new *KK* nucleus.

DISCUSSION

The results set forth above cannot be discussed in full without reference to observations and experiments not yet reported. A few of these will be briefly mentioned below but most will be reported in later papers. At this time, therefore, it is proposed merely to raise some of the problems connected with the preceding results and to point out some of the more important considerations bearing on them, reserving full discussion for later communications.

1. The Temporary Increase in Kappa Concentration After Fertilization.—The amount of kappa per cell is known to depend upon the relative rates of kappa and cell multiplication.[6, 7] Noting that animals with little kappa appear to experience a great rise in kappa concentration after fertilization, Sonneborn attributed this to continued multiplication of kappa at its usual

rate during the long delay in cell reproduction accompanying fertilization and subsequent nuclear reorganization. In like manner, the less marked but considerable increase in kappa concentration reported in this paper as reaching its peak at the 2nd fission after fertilization of animals containing initially much kappa may have a similar basis. Agreeing with this interpretation is the fact that the interval from the 1st to the 2nd fission after fertilization is distinctly longer than later interfission intervals, though not so much as the interval from fertilization to the 1st fission.

A possible weakness of the preceding explanation is that it fails to account for the decrease in kappa to its original level during the period between 2nd and 7th fissions, for these fissions take place at the same rate as the later fissions. It might be supposed that early rise of kappa concentration and its later fall are due to the early presence of more than the usual amount of macronuclear material (pieces of the old disintegrated macronucleus plus a new macronucleus) and the later restoration to the normal amount. This can hardly play a role here, however, for the same rise occurs when the new macronucleus is devoid of the gene *K* which controls kappa. Apparently other as yet unknown factors are operating here and experiments designed to discover them are now in progress.

2. The Loss of Kappa in the kk Cells Derived from Kk Killers at Autogamy.—The details of this loss raise a number of difficult questions. What determines the maintenance of kappa at the level characteristic of the parental *Kk* killers for 8 or more fissions? This cannot be ascribed alone to the store of the product of the gene *K*, although, as pointed out, enough of this is present or produced after fertilization to permit 10 or 11 doublings of the kappa in the *kk* cell. Yet the multiplication of kappa waits on cell growth and reproduction, as if some other factor essential for kappa is brought into the cell from the medium along with the food that supports cell increase.

What determines the sudden and rapid loss of kappa in some cells at each fission after the 8th while others retain it through the 14th fission? The meager data available do not fit well the interpretation that this is due to random distribution of a limited and unreplaced store of *K* product to the various cells. The proportions of cells losing kappa at each fission, as mentioned above and shown in table 2, are hardly the sort of results expected from random distribution; but more extensive data are clearly needed.

The maintenance of kappa in some cells through the 14th fission is at variance with Sonneborn's[6] inference that kappa disappears by the 6th fission. His conclusion, however, was based on the observation that reintroduction of gene *K* into these cells can result in maintenance of the killer trait only if the gene is reintroduced before the 6th fission. With this, the present observations are readily reconciled. As pointed out above, a new *KK* genotype appears to take 9 fissions to begin to come into action. Thus, introduction of gene *K* at the 6th fission might not be expected to

result in kappa increase until the 15th fission and by that time all the cells lack kappa. Thus, Sonneborn's observations may actually be understood as agreeing with the present work in showing that there is a 9-fission delay between introduction of a *K* gene and its earliest demonstrable action.

3. The Relation Between Dosage of Gene K and Cellular Concentration of Kappa.—A new feature has been added to the *K*-kappa system by the discovery that, among animals of the same mating type, homozygotes (*KK*) carry about twice as much kappa as heterozygotes (*Kk*). The gene *K* not only controls the maintenance of kappa, as has long been known, but further controls the quantity of kappa per cell. This relation between the dosage of a gene and the cellular concentration of a cytoplasmic particle provides a model, as Sonneborn[10,11] points out, of how gene mutation could raise the cellular concentration of a virus, thus affording a link between the mutation and virus theories of cancer.

So precise a numerical proportionality between dosage and gene effect is known in but few cases, the best known of which is the relation between vitamin A content and dosage of gene *Y* in the endosperm of maize.[12] The particulate nature of kappa, however, makes this case more striking.

4. The Basis for Mating Type Difference in Kappa Concentration.—The fact that, genotype for genotype, there are twice as many kappa particles in animals of mating type VII as in mating type VIII animals is discussed by Nanney[13] who finds in this observation a clue to the long-puzzling problems of the determination and inheritance of mating type.

Summary.—In both mating types and in both heterozygotes and homozygotes for the killer gene (*K*), the number of kappa particles per cell increases during first 2 cell divisions after fertilization to a level about three times as great as before fertilization. In the course of the following 4 or 5 fissions, it drops again to the level characteristic of the genotype and mating type, remaining at that level until the next fertilization. These characteristic kappa concentrations are approximately 200 for *Kk* type VIII cells, 400 for *KK* type VIII and *Kk* type VII cells, 800 for *KK* type VII cells. Change of genotype from *Kk* to either *KK* or *kk* results in no change from the *Kk* kappa concentration characteristic of this stage of the life cycle during the first 7 fissions. In the new *KK* cells, the kappa regularly rises to the *KK* level after the 9th and before the 10th fission. In the *kk* cells, the kappa suddenly drops to low levels or to zero after the 8th fission in some cells and progressively in more and more cells until the 15th fission when it disappeared from all. Apparently a newly introduced *K* gene regularly comes first to phenotypic expression 9 fissions later. The effect of previously present *K* genes disappears more irregularly, but starting at the 8th fission; meanwhile kappa multiplies even in *kk* animals.

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*MATING TYPE DETERMINATION IN PARAMECIUM AURELIA, A MODEL OF NUCLEO-CYTOPLASMIC INTERACTION**

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Sonneborn's studies[1] on mating types have shown two apparently different systems of determination and inheritance of mating types in the different varieties of *P. aurelia*. One system appears in the varieties designated as Group A (varieties 1, 3, 5, and 7) and the other in varieties designated as Group B (varieties 2, 4, 6, and 8). Most of the published data are on Group A, but Sonneborn has generously supplied unpublished data on Group B and these have been supplemented by further investigation. In spite of the large amount of accumulated data, it has been difficult to reconcile the Group A and Group B systems. The chief purpose of the present contribution is to supply a general hypothesis which can be applied equally well to both systems.

In order to develop such a general hypothesis, it is first of all necessary to summarize for the mating types and their determination and inheritance the known relations which are fundamental and common to both Groups A and B, and those which distinguish one group from the other. No more than two mating types occur in any variety and these are designated by an

odd and an even Roman numeral, a different pair of numerals being used for each variety. Although the varieties are effectively isolated, due to the non-viability which results from such intervarietal matings as occur, sufficient cross reactions are available to show for most varieties both that the odd mating types are similar but not identical and that the even mating types are similar but not identical.[2]

Sonneborn[1] cites a number of lines of evidence which make it clear that the macronuclei control the mating types, i.e., some unknown difference distinguishes the macronuclei in animals of the odd mating type from the macronuclei in animals of the even mating type. On the other hand, it is equally clear that no difference exists between the micronuclei in animals of different mating type. The chief unsolved problem is thus how to account for the existence of unlike macronuclei in animals containing identical micronuclei.

The main difference between Group A and Group B with respect to mating type determination and inheritance lies in the relationship between the mating type of a clone and the mating type of its descendent clones arising at the time of fertilization. After fertilization each cell gives rise to two new macronuclei which are separated at the first post-fertilization cell division. The descendents of each of these first fission products are called a caryonide and are in most cases alike in mating type. Sister caryonides produced from a single reorganized cell may, however, differ in mating type. In Group B varieties the sister caryonides are usually alike in mating type and have the same mating type as the parental clone, as a rule. The caryonides produced in an odd clone are nearly all odd in mating type; those from an even clone are predominantly even. This parent-progeny correlation has been shown[1] to depend upon different cytoplasmic conditions which are characteristic of the different mating types.

In varieties of Group A there is no such correlation between the mating types of the parents and of their sexually produced progeny. The same frequency of odd caryonides is produced at reorganization in odd and even clones. Any attempt to provide a general hypothesis must account for this difference between the Group A and Group B varieties.

Before attempting to account for this difference, a solution must be obtained to the prior and more general problem of the nature of the macronuclear differences. Earlier attempts to solve this problem will not be discussed, for they have either been discarded in view of later discoveries or have remained only in formal agreement with the facts and have lacked decisive experimental tests. The clue to the solution of this problem appears to be provided recently from an unexpected source, a study by Chao[3] of the killer trait in stock 51 of variety 4. As is well known,[1] the killer trait depends upon the presence in the cytoplasm of particles called kappa which in turn depend for their maintenance upon the nuclear gene K.

By direct count Chao finds: (1) a direct proportionality between the number of kappa particles in the cytoplasm and the number of K genes in the micronucleus; and (2) approximately twice as many kappa particles in animals of the odd mating type (VII) as in animals of the even mating type (VIII) when the animals have the same number of K genes in the micronucleus. This suggested to the present author the possibility that the macronuclei in the type VII animals contain twice as many K genes as do the macronuclei in the type VIII animals.

The suggested difference in the number of K genes in the macronuclei of the two mating types can be conceived in two ways. First, the chromosome bearing the K gene may be the only one present in different numbers in the macronuclei of animals of different mating types. Second, all the chromosomes may exist in different numbers in the two mating types. The decision between these alternatives is of considerable importance in determining the mode of action of the genes involved in both the killer trait and the mating type traits. An alteration in phenotype either quantitatively (as in the killer trait) or qualitatively (as is assumed in the mating types) due merely to an increase in the total number of chromosome sets in the cell is in clear conflict with the concept of genic balance. On the other hand, if the mating types differ only in the quantity of a single chromosome, as in the aneuploid interpretation, all the results found are apparently reconcilable with classical genetic theory. It should be pointed out, however, that the suggestion of mating type differences based on polyploidy is also founded on reasonable assumptions.[4]

The manner in which either of these interpretations is specifically applied depends upon the structure of the macronucleus. Again two general hypotheses are possible. Sonneborn[1] has given evidence that the macronuclei contain at least 30 to 40 pieces, each of which can regenerate into a macronucleus controlling the same hereditary traits as those controlled by the original macronucleus. Kimball[5] pointed out that these observations might be explained by assuming a very high degree of ploidy with random distribution of the chromosomes to the pieces into which the macronucleus disintegrates. On the other hand, Sonneborn suggested that the disintegration products represent discrete units of the original macronucleus and that each contains a balanced set of chromosomes. At the present time neither of these interpretations of macronuclear structure has been eliminated and both must be considered as possibilities.

Combinations of the polyploid-aneuploid and the organized-unorganized macronuclear interpretations yield a total of four sub-hypotheses which must be dealt with. Each of these yields somewhat different expectations. Studies to eliminate some of the possibilities are now in progress, but insufficient data are available to warrant full discussion at the present time. Rather, one of the possible interpretations has been chosen to dem-

onstrate the manner in which the general hypothesis can be applied. This subhypothesis hold that the macronuclei are composed of discrete subnuclei, each with balanced chromosome sets, and that the subnuclei in one mating type are diploid and those in the other mating type are tetraploid. Some of the reasons for preferring the hypothesis in this form will be indicated.

One of the problems related to mating type determination centers around the occasional cells, selfers,[6] which produce both mating types during vegetative growth. These cells can be interpreted on the above hypothesis as having macronuclei composed of some diploid and some tetraploid subnuclei which are segregated more or less randomly at cell division, giving some nuclei which are predominantly diploid and of one mating type, and some which are predominantly tetraploid and of the other mating type. Several lines of evidence are in agreement with this interpretation, at least in supporting the idea of localized intra-macronuclear differences. (1) Sonneborn[1] observed that mating type is inherited through any piece of the macronucleus. Therefore, any piece of the macronucleus contains all the necessary elements for mating type determination. (2) In selfing clones, different regenerated fragments from a single cell may in some cases control a pure mating type and in other cases a new selfing clone.[7, 8] This would be expected if some or all of the fragments contain two or more subnuclei. (3) Experimental fusion of macronuclei controlling different mating types results in the formation of selfing clones, even in stocks which normally do not produce them.[8] Although these lines of evidence do not constitute critical evidence against other interpretations, they are more readily understood on the basis of some kind of persistent submacronuclear differences.

A major problem is the manner in which the diverse macronuclei arise. On the basis of the present hypothesis diverse macronuclei arise through the doubling or the failure of doubling of the chromosomes in the unit nuclei which give rise to the new macronuclei. A doubling at the first intranuclear division of a subnucleus would produce a macronucleus composed entirely of tetraploid units; a doubling induced in only a part of the subnuclei at a later subnuclear division would give a mixed macronucleus, and a complete failure to double would produce a pure diploid macronucleus.

Sonneborn[1] has shown that the cytoplasm plays an important role in nuclear development in the Group B varieties. Specifically, macronuclei developing in the cytoplasm of a cell showing the odd mating type differentiate so as to control the odd type; nuclei developing in even type cytoplasm control the even mating type. In terms of the present hypothesis this would mean that cytoplasm in odd type cells is able to bring about a chromosome doubling while the cytoplasm in even type cells usually is not. To limit the discussion it may be assumed that this cytoplasmic activity is associated with a specific polyploidizing substance, *pl*. Since the micro-

nuclei in the two mating types are identical, it must be assumed that *pl* does not ordinarily act on the micronuclei. Since cells of either mating type can produce both types of progeny, but in different frequencies, it appears reasonable to conclude that the cytoplasmic differences between the mating types are quantitative and not qualitative.

Differences of opinion are possible in regard to the manner in which the cytoplasmic conditions are perpetuated. Sonneborn has interpreted the transmission of the cytoplasmic influence as due to a self-perpetuating cytoplasmic constituent. On the other hand, a formal explanation of the facts can be provided without resort to a self-perpetuating substance. Such an explanation rests on three assumptions: (1) the macronucleus itself produces *pl;* (2) a macronucleus with tetraploid subnuclei produces a larger amount of the substance than a macronucleus with diploid subnuclei; and (3) the quantity of *pl* formed in the cytoplasm of a diploid is usually insufficient to achieve polyploidization of the macronuclear anlagen, but the quantity formed in a tetraploid is nearly always adequate. At times of fertilization and nuclear reorganization the cytoplasmic product of the old macronucleus would affect the newly developing macronuclei so as to give them precisely that condition (diploidy or tetraploidy) which is required for maintaining the same concentration of the determinative cytoplasmic product.

These three assumptions have not been fully verified, but data are available which show that the macronuclei do control the condition of the cytoplasm to some extent. Whenever mating type changes, and hence when a different kind of macronucleus is formed, the cytoplasmic conditions associated with the different mating types usually change also. Particularly critical are those cases in which a single reorganizing cell produces two unlike caryonides with the same cytoplasmic source. When nuclear reorganization occurs in these caryonides, the progeny show the same mating type as the parental caryonide.[8]

The chief fact challenging this interpretation is that occasional caryonides (usually the even mating type) which have changed mating type at one reorganization can produce at a subsequent reorganization a high frequency of the other mating type.[7] Aside from the fact that such caryonides are much rarer than those in which the mating types of parents and progeny agree, the question has been raised as to whether many or most of such caryonides are not in fact selfers in which the frequency of one mating type is so low as to preclude detection with ordinary observation techniques. Often selfing caryonides which are predominantly of the even mating type will produce at reorganization a large fraction of odd type progeny.[8] This could be explained if a larger number of tetraploid subnuclei was necessary for the manifestation of the odd mating type than was necessary for building up the *pl* concentration to the point required for the determination of

new odd type macronuclei. If the occasional clones which revert at reorganization to a previous mating type are in fact selfers, and if this interpretation of selfers is correct, the chief reason for assuming a self-maintaining cytoplasmic substance is eliminated. This interpretation fails, however, to account for the rare caryonides of the odd mating type which revert and all the "unstable" even clones may not be selfers. Probably some as yet undetected factor is operating here and further work is required before a complete explanation is possible.

Even though it is clear that the macronucleus exercises an important influence on the cytoplasm, this does not disprove the existence of a self-perpetuating cytoplasmic substance; it only makes it unnecessary. At the present time no evidence is available distinguishing between nuclear control of a self-reproducing substance (as in the case of kappa) and the production of a substance directly by the nucleus. This latter interpretation is, however, preferred on the basis of simplicity and orthodoxy.

In Group B it is postulated that the concentration of *pl* in the cytoplasm is higher in tetraploid than in diploid animals. If, in Group A, the concentration of *pl* is independent of the level of ploidy in the macronuclear units, but is near the threshold concentration necessary for the induction of tetraploidy, there would be no correlation between the mating type of the parent and its sexually produced progeny. This is, of course, the situation observed. The condition postulated for Group A is in agreement with expectations on the theory of genic balance; the condition postulated for Group B is the remarkable and exceptional one, which is, however, supported by Chao's observations on kappa concentration.

There are then three results of polyploidy in the postulated scheme which are in disagreement with the concept of genic balance, the effect on kappa, the effect on mating types in both Group A and Group B, and the effect on the polyploidizing substance in Group B.

The justification for setting forth the as yet incompletely verified gene-dosage hypothesis of mating type determination and inheritance lies in the following considerations: The new interpretation can account formally for most of the available facts; it provides a fundamentally common interpretation for the two systems of mating type inheritance in the two groups of varieties; and it provides a broad working program upon which to base further experiments and tests.

Summary.—A general hypothesis of mating type determination in *P. aurelia* is proposed. This hypothesis holds that the physical basis of mating type differences lies in gene-dosage differences in the macronuclei of cells of different types. The achievement of the higher dosage levels (in the odd mating type) is due to a cytoplasmic substance produced under the influence of the macronucleus previously occupying the cell—the different types of macronuclei determining different mating types and different

amounts of cytoplasmic agent in the Group B varieties, but determining only different mating types in the Group A varieties.

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REPRESENTATIONS BY CUBIC CONGRUENCES

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In a paper to be published elsewhere,[1] the author obtains formulae for the number of solutions of a quadratic congruence relative to an arbitrary odd modulus, using an approach based on the theory of exponential sums. This method required nothing more than elementary properties of the quadratic Gauss sums. It is the purpose of this note to illustrate how such an approach can be applied to cubic congruences. We treat in detail two cases involving sums of three cubes relative to a prime modulus.

Let p be a prime, $p \equiv 1 \pmod 3$, and let χ_1 represent either one of the two non-principal cubic characters (mod p). With χ_1 fixed, denote the other non-principal character by χ_2, and the principal character by χ_0. We have $\chi_1^2 = \chi_2$, $\chi_2^2 = \chi_1$, $\chi_1\chi_2 = \chi_0$. We denote by $S(z, p)$ the cubic Gauss sum,

$$S(z, p) = \sum_{x=0}^{p-1} \epsilon(zx^3, p)$$

where

$$\epsilon(a, p) = e^{2\pi ia/p}.$$

We next place

$$\tau_1(z, p) = \sum_{(x, p) = 1} \chi_1(x)\epsilon(zx, p), \qquad \tau_1(1, p) = \tau_1,$$

$$\tau_2(z, p) = \sum_{(x, p) = 1} \chi_2(x)\epsilon(zx, p), \qquad \tau_2(1, p) = \tau_2,$$

both summations being over $0 < x < p$. The two τ sums are the cubic Gauss sums with characters (mod p).

We are interested in the function $N_s(\alpha, p)$ which is defined to be the number of solutions of the congruence

$$\alpha \equiv x_1^3 + \ldots + x_s^3 \pmod{p}.$$

In particular, we shall derive formulas for $N_3(1, p)$ and $N_3(0, p)$. To do this one needs only the following purely elementary facts concerning cubic Gauss sums:

$$N_s(\alpha, p) = \frac{1}{p} \sum_{z=0}^{p-1} \epsilon(z\alpha, p)(S(-z, p))^s. \tag{1}$$

This fact is an immediate consequence of the author's additive congruence principle.[2]

$$S(z, p) = \tau_1(z, p) + \tau_2(z, p), \qquad (z, p) = 1, \tag{2}$$

$$\tau_1\tau_2 = p, \ \tau_1^3 + \tau_2^3 = pA, \tag{3}$$

where A is the integer a of the uniquely determined pair (a, b), satisfying[3] $a \equiv 1 \pmod 3$, $4p = a^2 + 27b^2$, $b > 0$.

$$\tau_1(z, p) = \chi_2(z)\tau_1, \ \tau_2(z, p) = \chi_1(z)\tau_2, \ (z, p) = 1. \tag{4}$$

We use these properties to prove first[4]

THEOREM 1. *If α is a cubic residue (mod p), then*

$$N_3(\alpha, p) = p^2 + 6p - A.$$

Proof: It suffices to take $\alpha = 1$. Then by (1) and (2) we get

$$N_3(\alpha, p) = \frac{1}{p} \sum_{z=0}^{p-1} \epsilon(z, p)S^3(-z, p) = p^2 + \frac{1}{p} \sum_{z=1}^{p-1} \epsilon(z, p)(\tau_1(-z, p) + \tau_2(-z, p))^3.$$

Noting that $\chi_1(-1) = \chi_2(-1) = 1$, we have then by (4).

$$N_3(\alpha, p) = p^2 + \frac{1}{p}\sum_{z=1}^{p-1} \epsilon(z, p)(\chi_2(z)\tau_1 + \chi_1(z)\tau_2)^3$$

$$= p^2 + \frac{1}{p}\sum_{z=1}^{p-1} \epsilon(z, p)\{\tau_1^3 + \tau_2^3 + 3\tau_1^2\tau_2\chi_2(z) + 3\tau_1\tau_2^2\chi_1(z)\}$$

$$= p^2 + \frac{1}{p}\Big\{(\tau_1^3 + \tau_2^3)\sum_{z=1}^{p-1} \epsilon(z, p) + 3\tau_1^2\tau_2 \sum_{z=1}^{p-1} \chi_2(z)\epsilon(z, p) + 3\tau_1\tau_2^2 \sum_{z=1}^{p-1} \chi_1(z)\epsilon(z, p)\Big\}.$$

But the three sums have the values -1, τ_2, and τ_1, respectively, so that we get from (3)

$$N_3(\alpha, p) = p^2 - \frac{1}{p}(\tau_1^3 + \tau_2^3) + \frac{6}{p}\tau_1^2\tau_2^2 = p^2 - A + 6p.$$

We sketch now a proof of

THEOREM 2. $N_3(0, p) = p^2 + (p - 1)A$.

Proof:

$$N_3(0, p) = \frac{1}{p}\sum_{z=0}^{p-1} (S(-z, p))^3 = p^2 + \frac{1}{p}\sum_{z=1}^{p-1} (\chi_2(z)\tau_1 + \chi_1(z)\tau_2)^3 =$$

$$p^2 + \frac{1}{p}(\tau_1^3 + \tau_2^3)(p - 1) + 3\tau_1^2\tau_2 \sum_{z=1}^{p-1} \chi_2(z) + 3\tau_1\tau_2^2 \sum_{z=1}^{p-1} \chi_1(z).$$

The two sums are 0, so that the theorem follows from (3).

From Theorem 2 one may deduce the following familiar

COROLLARY. If α is a cubic residue (mod p), then

$$N_2(\alpha, p) = p - 2 + A.$$

Proof: We note the recursion

$$N_3(0, p) = (p - 1)N_2(\alpha, p) + N_2(0, p) = (p - 1)N_2(\alpha, p) + 3p - 2,$$

from which the corollary follows, on applying Theorem 2 and rearranging. This result may also be proved directly just as Theorem 1 or 2.

Although the above approach is applicable when α is a non-residue, the results are not quite so simple. A detailed account of this method applied to cubic congruences in two and three unknowns relative to a prime-power modulus will be given in a paper to be published elsewhere. Various other extensions are also possible.

[1] Cohen, E. (to appear in the *Duke Math. J.*).

[2] Cohen, E., *Duke Math. J.*, **19**, 115–129 (1952).

[3] Hasse, H., *Vorlesungen über Zahlentheorie*, Berlin, 1950, §20, 4b.

[4] Dickson, L. E., *Acta Arithmetica*, **1**, 161–167 (1935); in particular, see p. 167.

ALMOST PERIODIC FUNCTIONS ON LOCALLY COMPACT GROUPS

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1. Let G be a locally compact group and μ its left Haar measure. A family $\mathfrak{U} = \{U_t\}$ (t running over all positive numbers) of subsets of G is called a *left sampler family* on G, if it satisfies the following three conditions:[1]

(α) *For every $t > 0$, U_t is a non-empty open set with compact closure.* (This implies $0 < \mu(U_t) < +\infty$.)

(β) *For every $\varepsilon > 0$ and $y \in G$, there exists an $a > 0$ such that*

$$\mu(yU_t \Delta U_t) < \varepsilon \cdot \mu(U_t) \text{ for all } t \geq a.$$

(Here Δ denotes the symmetric difference: $A \Delta B = (A - B) \cup (B - A)$).

(γ) *For any $c > 0$ and $\varepsilon > 0$, there exists $b > 0$ such that for every $t \geq b$, there exists a finite subset $\{x_1, x_2, \ldots, x_n\}$ of G* (depending on t) *satisfying*

$$x_i U_c \cap x_j U_c = 0 \quad (i \neq j)$$

and

$$\mu(U_t \Delta \bigcup_{i=1}^{n} x_i U_c) < \varepsilon \mu(U_t).$$

Using this concept of left sampler family, we have:

THEOREM 1. *Let G be a locally compact group and μ its left Haar measure. If $\mathfrak{U} = [U_t]$ is a left sampler family on G, then for any measurable almost periodic function f on G, the limit*

$$\lim_{t \to \infty} \frac{1}{\mu(U_t)} \int_{U_t} f(x) d\mu(x) \tag{1}$$

exists. This limit is precisely the mean value of f in von Neumann's theory,[2] and hence is independent of the particular choice of the left sampler family $\mathfrak{U}$.

We first establish the following lemmas:

LEMMA 1. *For any $\varepsilon > 0$, there exists $c > 0$ such that*

$$\left| \int_{U_t} f(x) d\mu(x) - \int_{yU_t} f(x) d\mu(x) \right| < \varepsilon \mu(U_t) \tag{2}$$

for all $t \geq c$ and all $y \in G$.

Proof: By the almost periodicity[3] of f, there exists a finite covering $\{A_1, A_2, \ldots, A_n\}$ of G such that $|f(xz) - f(yz)| < \frac{\varepsilon}{2}$ for all $z \in G$, whenever x and y belong to the same A_i. Choose an element $x_i \in A_i$ $(1 \leq i \leq n)$. Let $\Gamma = \sup_{x \in G} |f(x)|$. By property ($\beta$) of $\mathfrak{U}$, we can find $c > 0$ such that

$$\mu(x_i^{-1}U_t\Delta U_t) < \frac{\varepsilon}{2\Gamma} \cdot \mu(U_t) \text{ for } t \geq c,\ 1 \leq i \leq n. \tag{3}$$

We claim that this c meets our requirement. In fact, consider an arbitrarily fixed $y \in G$. We can find an index i $(= 1, 2, \ldots, n)$ such that $|f(x_iz) - f(yz)| < \frac{\varepsilon}{2}$ for all $z \in G$. Then $|f(x) - f(yx_i^{-1}x)| < \frac{\varepsilon}{2}$ for all $x \in G$. Now the left-hand side of (2) is not greater than

$$\left| \int_{U_t} [f(x) - f(yx_i^{-1}x)]d\mu(x) \right| + \left| \int_{yx_i^{-1}U} f(x)d\mu(x) - \int_{yU_t} f(x)d\mu(x) \right|,$$

which is less than or equal to

$$\mu(U_t)\cdot\frac{\varepsilon}{2} + \Gamma\cdot\mu(x_i^{-1}U_t\Delta U_t).$$

Then by (3), we see that (2) holds for $t \geq c$.

LEMMA 2. *If V, W are two Borel sets of G such that $0 < \mu(V) < +\infty$, $0 < \mu(W) < +\infty$, then*

$$\left| \frac{1}{\mu(V)} \int_V f(x)d\mu(x) - \frac{1}{\mu(W)} \int_W f(x)d\mu(x) \right| \leq 2\Gamma \frac{\mu(V\Delta W)}{\mu(V)} \tag{4}$$

where $\Gamma = \sup_{x \in G} |f(x)|$.

Proof: The left-hand side of (4) can be written

$$\left| \frac{1}{\mu(V)} \left[\int_V f(x)d\mu(x) - \int_W f(x)d\mu(x) \right] + \frac{\mu(W) - \mu(V)}{\mu(V)\mu(W)} \int_W f(x)d\mu(x) \right|,$$

which is not greater than

$$\Gamma\frac{\mu(V\Delta W)}{\mu(V)} + \Gamma\frac{|\mu(W) - \mu(V)|}{\mu(V)}.$$

This together with $|\mu(W) - \mu(V)| \leq \mu(V\Delta W)$ gives (4).

Proof of Theorem 1: Let $\delta > 0$ be given. Choose $c > 0$ according to Lemma 1 for $\varepsilon = \delta/4$. For this c and $\varepsilon = \delta/8\Gamma$, we choose $b > 0$ according to property (γ). We claim that

$$\left| \frac{1}{\mu(U_r)} \int_{U_r} f(x)d\mu(x) - \frac{1}{\mu(U_s)} \int_{U_s} f(x)d\mu(x) \right| < \delta \tag{5}$$

holds for any $r \geq b$ and $s \geq b$.

Consider two arbitrarily fixed numbers $r, s \geq b$. According to our choice of b, there exist two finite subsets $\{x_1, x_2, \ldots, x_n\}$ and $\{y_1, y_2, \ldots, y_m\}$ of G such that:

$$x_iU_c \cap x_jU_c = 0 \quad (i \neq j) \tag{6}$$

$$\mu\left(U_r\Delta\bigcup_{i=1}^{n}x_iU_c\right)<\frac{\delta}{8\Gamma}\mu(U_r); \tag{7}$$

$$y_kU_c\cap y_lU_c=0\quad(k\neq l) \tag{8}$$

$$\mu\left(U_s\Delta\bigcup_{k=1}^{m}y_kU_c\right)<\frac{\delta}{8\Gamma}\mu(U_s). \tag{9}$$

From (6), we have

$$\frac{1}{n\mu(U_c)}\sum_{i=1}^{n}\int_{x_iU_c}f(x)d\mu(x)=\frac{1}{\mu\left(\bigcup_{i=1}^{n}x_iU_c\right)}\int_{\bigcup_{i=1}^{n}x_iU_c}f(x)d\mu(x).$$

Apply Lemma 2 and (7), we get

$$\left|\frac{1}{\mu(U_r)}\int_{U_r}f(x)d\mu(x)-\frac{1}{n\mu(U_c)}\sum_{i=1}^{n}\int_{x_iU_c}f(x)d\mu(x)\right|\leq$$

$$2\Gamma\frac{\mu\left(U_r\Delta\bigcup_{i=1}^{n}x_iU_c\right)}{\mu(U_r)}<\frac{\delta}{4}. \tag{10}$$

Similarly, we derive from (8), (9):

$$\left|\frac{1}{\mu(U_s)}\int_{U_s}f(x)d\mu(x)-\frac{1}{m\mu(U_c)}\sum_{k=1}^{m}\int_{y_kU_c}f(x)d\mu(x)\right|<\frac{\delta}{4}. \tag{11}$$

On the other hand, by our choice of c, we have

$$\left|\frac{1}{\mu(U_c)}\int_{U_c}f(x)d\mu(x)-\frac{1}{n\mu(U_c)}\sum_{i=1}^{n}\int_{x_iU_c}f(x)d\mu(x)\right|\leq$$

$$\frac{1}{n\mu(U_c)}\sum_{i=1}^{n}\left|\int_{U_c}f(x)d\mu(x)-\int_{x_iU_c}f(x)d\mu(x)\right|<\frac{\delta}{4} \tag{12}$$

and

$$\left|\frac{1}{\mu(U_c)}\int_{U_c}f(x)d\mu(x)-\frac{1}{m\mu(U_c)}\sum_{k=1}^{m}\int_{y_kU_c}f(x)d\mu(x)\right|<\frac{\delta}{4} \tag{13}$$

Clearly (5) follows from (10), (11), (12), (13). Thus the existence of the limit (1) is proved.

Let us denote by $\mathfrak{M}(f)$ the limit (1) and $M(f)$ the mean value of f in von Neumann's theory. To prove that $\mathfrak{M}(f)=M(f)$, it suffices to observe that, for any measurable almost periodic functions f, g on G and for any complex number α: (i) $\mathfrak{M}(f+g)=\mathfrak{M}(f)+\mathfrak{M}(g)$; (ii) $\mathfrak{M}(\alpha f)=\alpha\mathfrak{M}(f)$; (iii) $\mathfrak{M}(1)=1$; (iv) if f is real and ≥ 0 throughout G, then $\mathfrak{M}(f)\geq 0$;

(v) for any element $x_0 \in G$, $\mathfrak{M}(f(x_0x)) = \mathfrak{M}(f(x))$. [(v) is a consequence of Lemma 1.]

2. Let f be a complex-valued function defined on a locally compact group G. Let $\varepsilon > 0$. An element w of G is called an *ε-translation* of f, if $|f(xy) - f(xwy)| < \varepsilon$ holds for all $x, y \in G$. f is said to be *Bohr almost periodic*, if for every $\varepsilon > 0$ there exists a compact subset C of G such that zC contains an ε-translation of f for every $z \in G$.

According to this definition, for any compact group G, every complex-valued function on G is Bohr almost periodic. Hence there exist Bohr almost periodic functions which are not almost periodic (in the sense of von Neumann). However we have the following result:

THEOREM 2. *A complex-valued continuous function on a locally compact group G is Bohr almost periodic, if and only if it is almost periodic.*

We first prove the following lemmas:

LEMMA 3. *If f is an almost periodic function on a locally compact group G, then f is also Bohr almost periodic.*

Proof: Let $\varepsilon > 0$. Since f is almost periodic, there exists a finite covering $\{A_1, A_2, \ldots, A_n\}$ of G such that $|f(xuy) - f(xvy)| < \varepsilon$ for all $x, y \in G$, whenever u, v belong to the same A_i. For each i, let $w_i \in A_i$ $(1 \leq i \leq n)$. Then $C = \{w_1^{-1}, w_2^{-1}, \ldots, w_n^{-1}\}$ is a compact set. For any $z \in G$, there is an index i $(= 1, 2, \ldots, n)$ such that $z \in A_i$. Then $|f(xw_iy) - f(xzy)| < \varepsilon$ for all $x, y \in G$; or, what is the same, $|f(xy) - f(xzw_i^{-1}y)| < \varepsilon$ for all $x, y \in G$. Hence $zw_i^{-1} \in zC$ is an ε-translation of f.

LEMMA 4. *If f is a continuous Bohr almost periodic function on a locally compact group G, then f is uniformly continuous on G.*

Proof: Let $\varepsilon > 0$. Let C be a compact set in G such that zC contains an $\frac{\varepsilon}{3}$-translation of f for every $z \in G$. Let W be a neighborhood of the identity e in G such that its closure $\overline{W}$ is compact. Then f is uniformly continuous on the compact set $\overline{W}C$. Let V be a neighborhood of e such that $|f(y_1) - f(y_2)| < \frac{\varepsilon}{3}$ for any $y_1, y_2 \in \overline{W}C$ satisfying $y_1y_2^{-1} \in V$. We can assume $V \subset W$.

We claim that $|f(x_1) - f(x_2)| < \varepsilon$ for any $x_1, x_2 \in G$ satisfying $x_1x_2^{-1} \in V$. In fact, let w be an $\frac{\varepsilon}{3}$-translation of f such that $w \in x_2^{-1}C$. Then

$$|f(x_1) - f(x_2)| \leq |f(x_1) - f(x_1w)| + |f(x_1w) - f(x_2w)| + |f(x_2w) - f(x_2)| < 2\varepsilon/3 + |f(x_1w) - f(x_2w)|.$$

But $x_1w \in (Vx_2)(x_2^{-1}C) = VC \subset WC$, $x_2w \in C \subset \overline{W}C$ and $(x_1w)(x_2w)^{-1} = x_1x_2^{-1} \in V$; thus we have also $|f(x_1w) - f(x_2w)| < \frac{\varepsilon}{3}$. Hence $|f(x_1) - f(x_2)| < \varepsilon$.

Proof of Theorem 2: We need only prove that if f is a continuous Bohr almost periodic function on G, then f is almost periodic. Given $\varepsilon > 0$, we shall find a finite covering $\{A_1, A_2, \ldots, A_n\}$ of G such that $|f(ux) - f(vx)| < \varepsilon$ for all $x \in G$, whenever u, v belong to the same A_i.

Let C be a compact set in G such that xC contains an $\frac{\varepsilon}{3}$-translation of f for every $x \in G$. By Lemma 4, there exists a neighborhood V of e such that $|f(y_1) - f(y_2)| < \varepsilon/3$ whenever $y_1 y_2^{-1} \in V$. Let Z be a neighborhood of e that $ZZ^{-1} \subset V$ and $Z = Z^{-1}$. Let $\{x_1, x_2, \ldots, x_n\}$ be a finite subset of G such that $C \subset \bigcup_{i=1}^{n} x_i Z$. For each $i = 1, 2, \ldots, n$, let A_i be the union of all sets of the form wZx_i^{-1}, where w is any $\frac{\varepsilon}{3}$-translation of f.

We claim that $G = \bigcup_{i=1}^{n} A_i$. In fact, if $x \in G$, then xC contains an $\frac{\varepsilon}{3}$-translation w of f. Since $x^{-1}w \in C$, there is an index i such that $x^{-1}w \in x_i Z$. Then $x \in wZx_i^{-1} \subset A_i$.

Let u, v belong to the same A_i. Then $u \in w_1 Z x_i^{-1}$ and $v \in w_2 Z x_i^{-1}$ where w_1, w_2 are two $\frac{\varepsilon}{3}$-translations of f. For any $x \in G$, we have

$$|f(ux) - f(vx)| \leq |f(ux) - f(w_1^{-1}ux)| + |f(w_1^{-1}ux) - f(w_2^{-1}vx)| + |f(w_2^{-1}vx) - f(vx)|.$$

The first and third terms on the right-hand side is each $< \frac{\varepsilon}{3}$, as w_1, w_2 are $\frac{\varepsilon}{3}$-translations of f. The second term is also $< \frac{\varepsilon}{3}$, since $(w_1^{-1}ux)(w_2^{-1}vx)^{-1} \in ZZ^{-1} \subset V$. Hence $|f(ux) - f(vx)| < \epsilon$ for all $x \in G$.

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[1] For example, consider the group G of all matrices of the form $\begin{pmatrix} \xi & \eta \\ 0 & 1 \end{pmatrix}$ with $0 < \xi < +\infty$, $-\infty < \eta < +\infty$, with matrix multiplication as the group operation. If G is topologized in the obvious way as a subset (half-plane) of the Euclidean plane, then G is a locally compact group, whose left Haar measure μ is given by the Lebesgue integral $\mu(E) = \int\int_E \frac{1}{\xi^2} d\xi d\eta$ for any Borel set $E \subset G$. We obtain a left sampler family on G, if for every $t > 0$, U_t is the set of all matrices $\begin{pmatrix} \xi & \eta \\ 0 & 1 \end{pmatrix}$ such that $|\log \xi| < t$, $|\eta| < t$.

[2] Von Neumann, J., "Almost Periodic Functions in a Group, I," *Trans. Amer. Math. Soc.*, **36**, 445–492 (1934).

[3] We use Maak's definition of almost periodicity, which is equivalent to the definition of von Neumann-Bochner. See Maak, W., "Eine neue Definition der fastperiodischen Funktionen," *Abhandl. Math. Sem. Hans. Univ.*, **11**, 240–244 (1936).

ORGANISMAL DIFFERENTIALS AND ORGAN DIFFERENTIALS

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A series of transplantations of normal tissues and tumors which began in 1895, and was continued for a period of approximately fifty years, led to the concept that among various other substances functioning in a vertebrate organism, there is present in all, or almost all the tissues of an individual, among the higher organisms, a system of chemical substances, which is identical in the organs and tissues of the same individual, and which differs from that present in any other individual. In this system there is one particular substance which characterizes an individual, in contrast to a larger unit, such as a species. This chemical substance characteristic of an individual has been designated as his individuality differential. The character of the individuality differential is determined by and representative of the set of nuclear genes of this individual. The similarity and compatibility between the individuality differentials of the various individuals belonging to the same species depends upon the genetic relationship between these individuals. The pedigree relationship between members of a certain family also depends upon, and is an indicator of their genetic relationships, and in the analysis of genetic relationships, we may make use of family relationship and of phylogenetic relationships in general. In addition to the individuality differentials, there are present in each individual substances characterizing strains, races, species, genera and classes. Such a set of various substances represents the organismal differentials of an individual, or of a species.[1]

The individuality differential of an individual functions as an autogenous substance. The individuality differentials of other individuals in this species show different degrees of relationship to the first individual and and his differential. The individuality differentials of strange, not nearly related individuals, especially if they belong to a different strain, are homoiogenous in their relation to the first individual. Nearly related individuals belonging to the same family are characterized by syngenesious individuality differentials in their relationship to the first individual. The average of the syngenesio-differentials is more similar to the first individuality differential than is the average of the homoiogenous differentials, but within these various ranges (e. g., the syngensio-range) some special relationships exist according to the position in the family tree which these various individuals occupy.

A piece of normal tissue or tumor, when introduced into a host possessing a strange individuality differential, calls forth antagonistic reactions on the part of various types of host cells; the most characteristic of these is the

reaction of the lymphocytes of the host, which quantitatively indicates the degree of nearness or distance in the genetic relationship between host and transplant. It is the host which reacts against the transplant. Besides the lymphocytes, the connective tissue cells and blood vessels of the host react against bearers of strange individuality differentials. Furthermore, immune substances, which seem to be carried by the blood serum of the host, and, in all probability, also preformed substances present in the blood serum, may act against the strange organismal differentials. If the genetic relationship between host and transplant is as distant as that between two species or between distinct subspecies, a reaction on the part of polymorphonuclear leukocytes of the host partly replaces that of the lymphocytes. The mode and intensity of the reaction of the host cells is ultimately determined by the genes in the transplant, which are not represented in the host and are therefore strange to him. The reactions of a host are different if a piece of tissue from a hybrid offspring is introduced into one of the parents and if a piece from one of the parents is introduced into the hybrid offspring, a difference which was recognized in our early experiments. Notwithstanding these differences in the reactions in particular cases, it is justifiable to distinguish between the syngenesious and the homoiogenous relationship because both are characterized by different averages and by a different site in the spectrum of genetic relationships.

If families within the same species are continuously bred separately from one another, different strains develop. If the inbreeding in such families becomes very close as a result of continuous brother to sister mating, the individuals belong to such a strain may become more and more similar to one another in genetic constitution, although, contrary to the usual assumption, it seems to be very difficult to produce absolute identity between the individual differentials of the various members of a closely inbred strain.

In contrast to the organismal differentials which are the same in the different parts of an organism, we must distinguish the differentials of the various organs and tissues. Organs and tissues of an individual, though possessing the same organismal differentials, possess different organ and tissue differentials, based on their specific structure, metabolism and function, and especially on specific substances present intracellularly or given off to the surrounding body fluids. These organ and tissue differentials are very similar in different individuals; for example, the kidney and thyroid differential in one individual is very similar to the same organ differential in another individual. The organ and tissue differentials can be transferred either to a different site in the same individual or to a different individual, apparently without calling forth antagonistic reactions on the part of the host, except that under certain conditions immune reactions can also be produced against organs. Reactions which take place after transfer to a strange in-

dividual as a rule must be attributed to the strange organismal differentials which are associated with the organs and tissues. Thus, homoiotransplanted pieces of kidney and thyroid, although each differs greatly as to the organ-tissue differentials from the other, may elicit similar antagonistic reactions on the part of the host. However, the organ differentials may modify the action of the homoiogenous individuality differentials by influencing the cellularity and perhaps the activity of the metabolic processes of the transplant and thus they may influence the amounts of organismal differentials which are produced by the transplant and they may also affect the ready elimination of the organismal differentials and their action on the host; accordingly it might be expected that cartilagenous intercellular substances would be unfavorable to the ready discharge of the organismal differentials; moreover differences in the structure of the transplant might modify the resistance of the transplant to the antagonistic actions of the host. The organ-tissue differentials are also controlled by genes; but they are not identical with the genes which control the individuality differentials.

Organismal and organ-tissue differentials resemble each other in that both have undergone a phylogenetic as well as ontogenetic development; they both have progressed in a parallel way from simple to complex formations, ontogenetically and phylogenetically. However, this development took place in a somewhat different manner in these two types of differentials. The various organ-tissue differentials (e. g., those of kidney, thyroid, skin and liver) vary in different individuals, strains and species within a certain range independently of one another, and independently also of the organismal differentials associated with them.

Both organismal differentials and organ-tissue differentials have distinctive fields of function. In general, it is the function of the organismal differentials to maintain the integrity of the individual which they represent, to protect it against strange organisms or their component parts, against strange individuality differentials. At the same time, the identity of the individuality differentials and their autogenous nature, within the the same individual, prevent antagonistic reactions against the various constituents of its own body and makes them function as a harmonious whole. At least this is true as long as the different constituents of the body are approximately normal. Against strange individuality differentials the invaded host defends himself by means of the cellular reactions which take place around the strange material and presumably also by means of his own body fluids, at least under certain conditions. Strange organismal differentials serve readily as antigens and accordingly call forth the production of immune reactions. The initiation of immunity is therefore an important function of the organismal differentials. These reactions are the stronger the more distant the organismal differentials of the host are

from those of the invading organism; they are stronger against the bearers of strange species differentials than against the bearers of homoiogenous individuality differentials. It is therefore by means of strange organismal differentials serving as antigens that immunization against transplanted pieces of tumor possessing a different organismal differential is achieved. However, it is possible that, if the strength of the immunity produced differs in case different organs are used as antigens for the immunization of the host, organ-tissue differentials may play an additional role as antigens.

Early investigations on transplantation of tumors proved the existence of immune reactions against the strange organismal differentials which they carried, and we also noted the development of immunity against transplanted tumors which were bearers of individuality differentials differing from those of the host. We believed that similar immunizations might be expected to develop also against transplants of normal tissues which carried strange individuality differentials, but our early attempts to prove the existence of such immune or sensitizing reactions by means of the lymphocytic reaction did not yield a positive result; a second transplantation into a host which should have been immune as the result of a preceding transplantation of related tissue was not followed by an earlier or more intense lymphocytic reaction. However, subsequent experiments carried out in our laboratory by H. T. Blumenthal[2] proved that a second transplantation of normal tissue led to an earlier appearance of lymphocytes in the blood, although the number of lymphocytes was not increased. The immune reaction was therefore weak, and this suggested that the body fluids might also participate in the injury of the transplant by means of preformed substances which they carried. The recent experiments of Kidd[3] showed that immunity against transplanted Brown-Pearce carcinoma in the rabbit depends upon immune substances developing in lymphoid organs and that the immunized lymphocytes injured by direct action the strange tumor cells; on the other hand, in the blood serum of the host, the presence of immune substances could not be demonstrated. This latter observation is in agreement with the negative results of earlier attempts to demonstrate the presence of antibodies against strange transplanted tumor in the blood serum. The experiments of Kidd were carried out with homoiogenous tumor. It is conceivable that immunization against different species might permit the observation of antibodies also in the blood serum of the host.

Very different from the functions of the organismal differentials are those of the organ-tissue differentials. The latter as such and in their interaction which other organs make possible the normal life of the individual and its principal pathological deviations. In particular, they are also responsible for the reproductive processes of the individual and for his psychical manifestations which latter are dependent on the function of the nervous system within the whole organism. There is ordinarily an interaction between

the organ and tissue specificities and various environmental factors. By means of the psychical processes, the individual creates a picture of his personality which he attempts to elevate in the social struggle with other individuals. The psychical individuality differs from that essentially based on the function of the organismal differentials in that the former is amenable to the influence of psychical factors, such as thoughts, emotions and suggestions.

Likewise, various abnormal phenomena, such as growth processes, and in particular the development of tumors are the expression of changed organ functions; and although these changes may vary in accordance with differences in genetic relationships of individuals, strains, and species, they are not essentially the expression of organismal, but of organ differentials, which latter vary in accordance with family, strain, species characteristics. Also, various reactions of cancerous tissue are the expression of the organ-tissue differentials of the cancer or of the tissue from which it originated.

As the result of close inbreeding by consecutive brother to sister matings in families belonging to the same species, all individuals belonging to the strains thus developing become more and more similar, without, however, as a rule reaching identity in their genetic constitution and a completely autogenous state. The increasing similarity resulting from close inbreeding applies to both organismal differentials and organ-tissue differentials. If matings were not limited to brothers and sisters, but took place also between less nearly related individuals, the homogeneity in the reactions of the members of such families became less great; but the reactions in closely inbred families and in less closely inbred families differed only in degree and various intermediate stages did develop. It has therefore been possible to recognize some of the important characteristics of tumors and also of normal tissues in the course of the early study of animals affected by cancer preceding the time when very closely inbred strains were available for research; these characteristics may serve as examples for various other kinds of reactions that may take place under such conditions. Thus as far as the organismal differentials are concerned, it was recognized at these early periods that the lymphocytic reaction corresponds to the genetic relationship between host and transplant and to the relationship of their individuality differentials. This implies that the genetic constitutions of both host and transplant (donor) control the reactions of the host.[1]

Furthermore, the reactions of the host were found to be more severe when tissues of the hybrid offspring were transplanted into the parents than when tissues of the parents were transplanted into the offspring. Likewise, the theory of the individuality differentials implied, and it was subsequently also stated by Loeb and Wright[4] that the reaction of the host against the transplant does not depend upon the presence of a given number of factors in the transplant, but that the host reacts against the genes strange to him

which are present in the transplant. In addition it was noticed in this early period that successive transplantations of pieces of tumor within the same species as a rule increased the growth energy and decreased the latent period of the transplant.[5] We attributed this effect to mechanical stimulation of the transplant which occurred during the process of transplantation rather than to a loss of genes in the transplant; no change in the constitution of the individuality differential of the latter was involved.

Our early observations also noted the preponderance of the influence on the mother in the development of mammary carcinoma in the hybrid offspring in mice, if both parents differed in their genetic tendency in respect to the formation of this tumor.[6] In the case of mammary cancer of mice it was likewise established at this time that its appearance does not depend solely upon the nature of the organ-tissue differential in an individual belonging to a certain strain, but upon the interaction of this differential with hormones and it was suggested that a similar interaction between organ differentials and hormones might apply also in the case of abnormal growth processes in other organs.[7] In a few cases in which surgeons had unsuccessfully attempted to treat mammary carcinoma in patients by ovariectomy, no thought seems to have been involved as to the important role which hormones may play in the origin of cancer.

We may briefly mention some other observations made at this early period when not yet closely inbred strains were used in the transplantation of normal tissues or tumors, and in the experimental study of the development of cancer in certain organs. Thus, we observed the relationship between the incidence of tumors and the length of the latent period in the formation of mammary carcinoma in mice.[8] Likewise the action of multiple factors in the transplantation of tissues and in the development of tumors was recognized, the occurrence of many intermediate degrees of tumor incidence in certain families and strains was established, and the effect of breeding and pregnancies in increasing the incidence of mammary carcinoma in mice and the occurrence of variations in the strength of this influence in different strains also was noted.[1, 9] Observations were made concerning the effects of hybridization on the tumor incidence and concerning a possible relationship between hair color and the incidence of tumors. It was also noted that animals bearing a spontaneous tumor were a more favorable soil for transplantation of another spontaneous tumor that had developed in a homoiogenous animal.[10] The occurrence of a competitive struggle between adjoining tissues if they are affected by certain stimulations, either hormonal or mechanical, was also recorded at this time.[11]

During the process of close inbreeding of a strain, the sets of genes of the different members of the strain change from unlikeness more and more in the direction toward identity of distribution and in accordance with this change in the assortment of genes the reactions of the different organisms composing

the strain and of their constituent organs and tissues also undergo changes and become more and more alike in the different members of the strain. These changes in the individuals and in the various reactions of the organs of these individuals presumably take place at different stages in this process of inbreeding. The results observed depend also on the degree of relationship of these characteristics to factors in the inner or outer environment. The more these changes depend on environmental factors, the less readily do the reactions among different individuals tend to become identical, and it is merely the averages in the modes of reaction which approach identity. On the whole in their development the organismal differentials seem to be more independent of the environment than are various organ and tissue differentials, which may interact not only with variable environmental factors, but also with the organismal differentials. Thus, whereas in a closely inbred strain, the organismal differentials may approach identity in the various individuals as shown by transplantation experiments, the reactions of various organs as those of the mammary gland may vary greatly in individuals belonging to the same strain, as indicated by the individual variations in the development of tumors and in some of the other reactions which we mentioned.[8] In these cases only the averages in the reactions of the organs may become increasingly alike in the various individuals of the inbred strain. Therefore, with the continued progress in inbreeding, the similarity in the reactions which depend on the organismal differentials becomes increasingly greater until in the end they approach identity in different individuals, in contrast to the reactions which depend on organ-tissue differentials when the individual variations may remain greater.

It will be the task of further investigations to continue the study of the interactions between organismal and organ-tissue differentials among themselves and with environmental factors.

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